November 22, 2000

Dockets Management Branch (HFA-305)
Food and Drug Administration
5630 Fishers Lane, Rm. 1061
Rockville, Maryland 20852

Re: Docket Nos. 00P-1275 and 00P-1276
Food Labeling: Health Claims; Plant Sterol/Stanol Esters and Coronary Heart Disease (Interim Final Rule)
65 Fed. Reg. 54686 (September 8, 2000)

To Whom It May Concern:

Lipton, a Unilever company, submits the following comments on the above-referenced interim final rule.

COMMENT 1. The interim final rule was adopted in part in response to our petition for a health claim for plant sterol esters. We appreciate the FDA's careful review of our petition, and particularly appreciate the agency's permitting prompt use of the health claim in labeling through the promulgation of an interim final rule. We commend the agency for its effective action in fulfilling the goal of the law – to assist consumers in maintaining healthy dietary practices.

COMMENT 2. We concur with the FDA's determination that 1.3 g/d of plant sterol esters has consistently been shown to lower blood total and LDL cholesterol. 65 Fed. Reg. 54703-704; 21 CFR 101.83(c)(2)(i)(G)(*1*).

COMMENT 3. We request that the FDA reconsider requiring the health claim to include a statement that the daily dietary intake of plant sterol esters should be consumed in two servings eaten at different times of the day with other foods. Instead of requiring this "twice-a-day" language to be a mandatory part of the health claim, we urge the FDA to permit an optional statement recommending daily consumption of plant sterol ester-containing foods. This request is made principally to ensure that the regulation for plant sterol esters is consistent with other health claim regulations, and that manufacturers have appropriate flexibility in recommending consumption levels in labeling.

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Other health claim regulations do not mandate the use of labeling that specifies a required number of servings per day. For example, in the case of psyllium, the agency concluded that consumption multiple times a day was desirable and it targeted a consumption level of four times per day. However, it did not require the label to state, "eat four times a day." 63 Fed. Reg. 8109-10, 21 CFR 101.81(c)(2)(i). Similarly, in the case of soy protein, the agency targeted a consumption level of four times per day but did not require the label to state, "eat four times a day." 64 Fed. Reg. 57713-14 (Oct. 26, 1999); 21 CFR 101.82(c)(2)(i). For these health claims, the agency followed 21 CFR 101.14(d)(2)(vii) by requiring the claim to state the daily dietary intake of the substance necessary to achieve the claimed effect. That is, a health claim is required to refer to the daily amount of psyllium or soy protein associated with reduced risk of coronary heart disease, and the contribution one serving of the food makes to that daily amount.

Like the psyllium and soy protein health claim rules, the sterol ester rule requires that the claim refer to the daily dietary intake of sterol esters associated with reduced risk of coronary heart disease (i.e., 1.3 g or more per day), and the contribution that one serving of the product contributes to that level. 21 CFR 101.83(c)(2)(i)(G). As with the psyllium and soy protein regulations, this information is adequate to inform consumers about how to meet the daily intake level. That is, for foods that contain 0.65 g sterol esters per serving, this information informs consumers of the need to eat at least two servings a day. The extra step of requiring "twice-a-day" labeling in section 101.83(c)(2)(i)(H) is not necessary to achieve this goal. "Twice-a-day" labeling can be optional, but it need not be mandatory.

We understand that the FDA included the "twice-a-day" labeling requirement because most of the studies on which the claim is based involved consumption of plant sterol esters at least twice a day with other foods. 65 Fed. Reg. 54704-705. In our view, however, these studies show such consistent cholesterol-lowering results that they indicate that plant sterol esters will reduce cholesterol irrespective of whether they are consumed one or more times per day. This is corroborated by data from a recent study of stanol esters (whose mechanism of action is similar to that of sterol esters[1]) in which intake once a day was compared to intake multiple times a day and no significant difference in cholesterol lowering effect was observed.[2] Thus, we believe that twice-a-day consumption need not be mandatory in order to obtain the intended effect, and therefore – although it may be appropriate to optionally recommend multiple servings a day – it is not necessary to require them.

Indeed, there is a risk that a mandatory statement about "twice-a-day" consumption may discourage consumers from using the product. It is well recognized that dietary

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[1] Normen, L. et al.: Soy sterol esters and beta-sitostanol ester as inhibitors of cholesterol absorption in human small bowel. Am. J. Clin. Nutr. 2000;71:908-913. Jones, P.J. et al.: Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. J. Lipid Research 2000;41:697-705. (Copies attached.)

[2] Plat, J. et al.: Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters. Eur. J. Clin. Nutr. 2000;54:671-677. (Copy attached.)

modification to achieve cholesterol reduction is most effective when it requires the least disruption to normal behavior. If the health benefit of the product is conditioned on twice-a-day consumption, this may unnecessarily discourage use among consumers who wish to eat the food as they normally would without modifying their diet. Thus, although it may be appropriate to optionally recommend multiple servings a day, it is not appropriate to require them.

In addition, some products may be formulated with at least 1.3 g sterol esters per serving. For these products, it would be inappropriate to suggest that the claimed health benefit cannot be achieved without twice-a-day consumption (although it would be appropriate to optionally recommend multiple servings per day).

Finally, we note that the FDA's requirement that the label refer to consumption "with other foods" is unnecessary for spreads or dressings, which are always eaten with other foods.

In conclusion, we request that the FDA delete the requirement for "twice-a-day" labeling in section 101.83(c)(2)(i)(H) because the sterol ester health claim is complete, truthful, and not misleading without it, as required by 21 CFR 101.14(d)(2). In place of this mandatory language, we request that the FDA revise 21 CFR 101.83(d) to permit an optional statement recommending multiple servings per day of plant sterol ester-containing foods. Such a recommendation could vary from food to food, as appropriate, and might include, for example –

- for products formulated with at least 0.65 g per serving, "Eat two servings daily with meals."
- for products formulated with at least 1.3 g per serving, "Eat one serving daily with meals."

COMMENT 4. In promulgating the interim final rule on plant sterol esters, the FDA said that it would consider broadening the categories of foods eligible to bear the health claim if comments "submit supporting data establishing that the use of plant sterol esters in other food products is safe and lawful and provide a validated analytical method." 65 Fed. Reg. 54707-708. We agree with these requirements, and in particular we urge the FDA to consider the following before broadening the categories of foods eligible to bear the claim:

a. Any new use of plant sterol esters should not exceed the acceptable daily intake as established in the published literature or publicly available information.

b. Any new use of plant sterol esters should be consistent with the currently approved use, that is, plant sterol esters dissolved in the fat or oil phase of the food. Since the currently approved use was based on studies of sterol esters in fat-based foods, extending the health claim to foods that are not fat-based may

need to be supported by new studies demonstrating effective cholesterol reduction.

c. Any new use that involves an exemption from the limit for total fat in 21 CFR 101.14(a)(4) or the limit for minimum nutrient contribution in 21 CFR 101.14(e)(6) should be based on documented justification for the exemption.

Thank you for your consideration of these comments.

Respectfully submitted,

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Soy sterol esters and β-sitostanol ester as inhibitors of cholesterol absorption in human small bowel [1–3]

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ABSTRACT

Background: Plant sterols are natural dietary components with serum cholesterol–lowering properties. The lowering of serum cholesterol by plant sterols is believed to be the result of an inhibition of cholesterol absorption in the small bowel, although increased bile acid excretion has also been suggested. The difference in effect of saturated and unsaturated plant sterols on cholesterol absorption needs to be elucidated further.

Objective: The primary aim of this study was to measure small-bowel cholesterol absorption and sterol excretion in addition to hepatic cholesterol synthesis after intake of soy sterol esters and β-sitostanol ester corresponding to 1.5 g plant sterols/d.

Design: Seven ileostomy subjects were studied during a control period and 2 intervention periods when either soy sterol esters or β-sitostanol ester was added to a basal diet. Ileostomy bags were collected every other hour and frozen immediately for analysis of nutrients and sterols.

Results: Cholesterol absorption was 56% (43–65%) in the control period and decreased to 38% (32–46%) in the soy sterol ester period ($P = 0.00$) and to 39% (30–48%) in the β-sitostanol ester period ($P = 0.00$).

Conclusion: Esterified soy sterols and β-sitostanol inhibited cholesterol absorption equally, despite the different structures of the plant sterols. *Am J Clin Nutr* 2000;71:908–13.

KEY WORDS Plant sterols, phytosterols, plant sterol ester, cholesterol absorption, cholesterol synthesis, lathosterol, sterol excretion, ileostomy

INTRODUCTION

Plant sterols are natural dietary components with serum cholesterol–lowering properties (1). The most common plant sterols are β-sitosterol, campesterol, and stigmasterol, which are classified as 4-desmethylsterols of the cholestane series (2). The structures of these plant sterols are similar to that of cholesterol with an extra methyl or ethyl group and a double bond in the side chain. Saturated plant sterols, referred to as stanols, have no double bond in the ring structure (3). As early as 1951 it was shown that β-sitosterol decreased serum cholesterol in chickens during a cholesterol load (4). This finding resulted in several studies of the cholesterol-lowering effects of plant sterols in humans (1, 5–12).

The serum cholesterol–lowering effect of plant sterols is believed to be caused by an inhibition of cholesterol absorption resulting from the higher affinity of plant sterols than of cholesterol for micelles (13). Additionally, specific plant sterols may increase bile acid excretion (9). Plant sterols seem more efficient as serum cholesterol–lowering agents when mixed with fat than when alone (11). Most studies have been performed with free sterols, which have a low solubility in fat and therefore a limited ability to dissolve in butter or margarine. Esterification of free plant sterols is one way to increase solubility in fat (14). A clinical study of hypercholesterolemic subjects concluded that esterified β-sitostanol was more efficient than free β-sitosterol, free β-sitostanol, or rapeseed-based margarine alone in lowering serum cholesterol (14). The superiority of β-sitostanol ester has yet to be confirmed because the intakes of the dietary plant sterols were different in the experimental groups of the trial. A comparison of free β-sitostanol with free β-sitosterol, however, showed that the saturated plant sterol increased cholesterol excretion more effectively than the unsaturated plant sterol when infused over several hours in low concentrations (3). Despite the latter finding, a recent comparison of esterified unsaturated sterols from soybeans with the ester of the saturated β-sitostanol indicated that soybean sterol esters had a similar serum cholesterol–lowering effect as the β-sitostanol ester (12). It could therefore be hypothesized that soy sterols and β-sitostanol inhibit cholesterol absorption equally when both fractions are esterified.

The conventional sterol balance technique can be criticized for being imprecise when used to measure cholesterol and bile acids because of the considerable variation in colon transit time and bacterial degradation (15). To improve the precision of measurement of small-bowel excretion, sterol excretion can be studied in ileostomy subjects. Analysis of ileostomy bags after immediate freezing reveals an almost complete absence of sec-

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[2] Supported by grants from the Swedish Cancer Foundation and the Swedish Nutrition Foundation. Plant sterol esters were generous gifts from the Raisio Group, Raisio, Finland.

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ondary bile acids, which might correspond with a low rate of fermentation (16). The primary aim of this study was to measure small-bowel cholesterol absorption and sterol excretion and hepatic cholesterol synthesis after the intake of soy sterol esters and β-sitostanol ester corresponding to 1.5 g plant sterols/d.

SUBJECTS AND METHODS

Subjects

Seven ileostomy subjects, 5 men and 2 women, were studied. All subjects had undergone proctocolectomies for ulcerative colitis and had well-functioning ileostomies. The median (range) time since surgery was 13 y (2–26 y). The subjects had a median body mass index (BMI; in kg/m^2) of 26 (18–30) and a median age of 54 y (29–73 y). Three subjects took medications for hypertension, 1 subject took medication for hypothyroidism, and 1 subject took medication for sacroiliitis. The subjects were otherwise healthy and showed no signs of anemia, inflammation, or hepatic or thyroid disease as confirmed by history and standard laboratory tests. An inclusion criterion for subjects was a total bile acid excretion of <1 g/d. Higher bile acid excretion reflects a large resection of the small bowel, which would break the normal enterohepatic circulation of bile acids. Informed consent was obtained from all subjects and approval for the study was granted by the ethics committee of Göteborg University in February 1996.

Design

The study consisted of 1 control period and 2 intervention periods of 3 d each. The order of periods was assigned randomly and the subjects completed the study over 3 consecutive weeks. There was a minimum washout period of 4 d between every study period. A basal diet was served during all 3 periods. During the intervention periods, 2.5 g soy sterol ester or β-sitostanol, corresponding to 1.5 g free plant sterols, was added to the basal diet daily. The first day of every period was an adaptation day to minimize carryover effects from the subjects' habitual diets. On study days 2 and 3, the subjects changed ileostomy bags every second hour. The night bag was collected separately. Bags were frozen on dry ice immediately to minimize bacterial degradation. Collection started at 0730 with a change of the first bag at 1000 and continued until 2200. Each subject's bags were freeze-dried, pooled (24 h), and stored at −20°C until analyzed for energy, nitrogen, starch, resistant starch, dietary fiber, and sterol contents.

A blood sample was drawn before the start of the study to determine each subject's apolipoprotein (apo) E phenotype. On the third day of each period, a blood sample was drawn while subjects were in a fasting state to measure serum concentrations of plant sterols and the hepatic cholesterol synthesis marker lathosterol.

Basal diet and plant sterols

The basal diet was designed to have a low content of plant sterols and a high content of cholesterol (Table 1). The high cholesterol intake was chosen so that there would be enough cholesterol for the plant sterols to inhibit. Food items with a known high cholesterol concentration were chosen, eg, eggs, milk products, and meat. The composition of the diet was calculated from Swedish national food composition tables (17) and a computer program was used for nutrient calculations (DIETIST; Näringsdata AB, Bromma, Sweden). Analyses of starch, resistant starch, fiber, and sterols were performed on duplicate portions of the diet. Breakfast was served in the study center every morning of the 3 experimental days. The remaining meals were provided by the study center for subjects to consume at home or at work.

The basal diet consisted of breakfast (0730), fruit (1000), lunch (1200), coffee (1500), dinner (1700), and an evening snack (2000). All foods were prepared from the same batches and were stored in special containers at −20°C. Subjects were allowed to eat the prepared food only. Leftovers were weighed and recorded each day. Subjects drank tap water but were asked to drink the same amount at the same time every day.

The daily amount of plant sterol esters was divided into 3 portions, which were weighed directly onto small buns spread with butter. The buns were eaten at breakfast, lunch, and dinner. The 2.5 g soy sterol esters contained 722 mg β-sitosterol, 408 mg campesterol, 225 mg stigmasterol, 50 mg Δ-5-avenasterol, 38 mg brassicasterol, 20 mg campestanol, and 8 mg Δ-7-stigmasterol, which gave a total sum of 1.47 g plant sterols. The 2.5 g β-sitostanol ester contained 1363 mg β-sitostanol, 119 mg campestanol, 25 mg β-sitosterol, 8 mg campesterol, and 2 mg brassicasterol, resulting in a total dose of 1.52 mg plant sterols. The soy sterols were purchased from Archer Daniels Midland (Decatur, IL). The source of the β-sitostanol was tall oil (The Raisio Group, Raisio, Finland). Transesterification of both plant sterol fractions with oleic acid from rapeseed oil was performed by The Raisio Group. The esterification rates were 94% for soy sterols and 98% for β-sitostanol and the purity of both products was >95%.

Cholesterol absorption

Cholesterol absorption in the small bowel was measured with ^{3}H-labeled cholesterol and ^{14}C-labeled β-sitosterol according to a modification of the method of Grundy (18). On the second day of each study period a total dose of 3.9 kBq (106 nCi) [β-4-^{14}C]sitosterol and 8.6 kBq (233 nCi) [1α, 2α-n-^{3}H]cholesterol dissolved in 750 mg rapeseed oil was weighed onto 3 sugar cubes. The sugar cubes were served with the same meals as the

TABLE 1
Composition of the basal diet

	Value
Energy (MJ)[1]	10.0
Total fat (% of energy)[1]	37
Protein (% of energy)[1]	17
Carbohydrates (% of energy)[1]	45
Starch (g)[2]	102
Resistant starch (g)[2]	5
Dietary fiber (g)[2]	14
Sterols (mg)[2]	
Cholesterol	775
Total plant sterols	172
β-Sitosterol	94
β-Sitostanol	13
Campesterol	43
Campestanol	7
Brassicasterol	30
Stigmasterol	10
5-Avenasterol	5

[1] Calculated from the Swedish Food Composition Tables (17).
[2] Analyzed value.

plant sterol–fortified buns. β-Sitosterol was purchased from Amersham International (Buckinghamshire, United Kingdom) and the tritium was from Duomedical (Stockholm).

Analytic procedures

The energy content of the basal diet was analyzed by bomb calorimetry (automatic adiabatic bomb calorimetry; Gallencamp, Loughborough, United Kingdom). Nitrogen content was determined by a modified micro-Kjeldahl method (19). Total starch and resistant starch were analyzed by the modified enzymatic procedure of Englyst et al (20) and dietary fiber was measured by the method of Asp et al (21). Radioactive isotopes of β-sitosterol and cholesterol in the ileostomy effluents were extracted according to the method of Miettinen et al (22). The samples were analyzed in a Beckman Tri-Carb liquid scintillation counter (model 1900 TR; Packard Instruments, Meriden, CT) with automatic external standardization. Recoveries of isotopes added to the diet in ileostomy excreta were 92% for β-sitosterol and 89% for cholesterol, which was taken into account during the calculations of cholesterol absorption. Plant sterols were analyzed according to the method of Jonker et al (23), with some modifications (24, 25). In short, the method is based on acid hydrolysis, alkaline hydrolysis, silylation with trimethylsilylether, and finally gas-liquid chromatography (24). Sterols in the ileostomy excreta were analyzed as described by Bosaeus and Anderson (16). A cholesterol precursor, lathosterol, and plant sterols were analyzed in the nonsaponifiable part of serum by gas-liquid chromatography (26). Phenotyping was performed according to the method of Johansson et al (27). Serum cholesterol, HDL, and triacylglycerols were analyzed by an enzymatic procedure developed by Bayer Technicon AB (Stockholm).

Calculations and statistics

LDL-cholesterol concentrations were calculated with the Friedewald formula (28). The Quintao formula was used to calculate fractional cholesterol absorption as follows (29):

$$\text{Fractional cholesterol absorption (\%)} = 100 \times (1 - [(\text{fecal } [^3\text{H}]\text{cholesterol}/\text{fecal } [^{14}\text{C}]\beta\text{-sitosterol})/(\text{administered } [^{14}\text{C}]\beta\text{-sitosterol}/\text{administered } [^3\text{H}]\text{cholesterol})]) \quad (1)$$

Results are presented as medians and ranges in the text and tables. Statistical analyses were performed with the statistical package SYSTAT for WINDOWS (version 7.0; SPSS Inc, Chicago). Cholesterol absorption, ratios of lathosterol to cholesterol, and cholesterol and bile acid excretion were analyzed by analysis of variance. Significantly different pairwise comparisons were identified by a one-sided Dunnett test. Adjustment was made for multiple comparisons according to Bonferroni. $P < 0.05$ was chosen for statistical significance.

RESULTS

Before the beginning of the study, the subjects' median serum cholesterol concentration was 5.2 mmol/L (4.9–6.5 mmol/L) and their median LDL-cholesterol concentration was 3.2 mmol/L (2.6–4.0 mmol/L). Serum triacylglycerol concentrations were 0.99 mmol/L (0.81–2.0 mmol/L), LDL concentrations were 3.9 mmol/L (3.1–5.0 mmol/L), and HDL concentrations were 1.5 mmol/L (1.2–2.1 mmol/L). The individual subject's apo E phenotypes were as follows: E4,3 (subject 3); E3,3 (subjects 1, 2, 4, and 6); E3,2 (subject 5); and E2,3 (subject 7).

On day 3, the subjects had median serum β-sitosterol concentrations of 8.0 μmol/L (4.7–13.1 μmol/L) during the control period, 8.6 μmol/L (4.7–15.2 μmol/L) during the soy sterol ester period, and 9.6 μmol/L (6.4–17.1 μmol/L) during the β-sitostanol ester period. Median serum campesterol concentrations were 12.5 μmol/L (8.2–19.3 μmol/L) during the control period, 14.3 μmol/L (10.2–29.7 μmol/L) during the soy sterol ester period, and 14.2 μmol/L (10.6–20.9 μmol/L) during the β-sitostanol ester period. On day 3, the subjects had median serum concentrations of total cholesterol of 5.5 mmol/L (4.4–7 mmol/L) during the control period, 5.3 mmol/L (4.4–7.2 mmol/L) during the soy sterol ester period, and 6 mmol/L (4.5–7.2 mmol/L) during the β-sitostanol ester period.

Cholesterol absorption was 56% (43–65%) in the control period and decreased to 38% (32–46%) in the soy sterol ester period and to 39% (30–48%) in the β-sitostanol ester period ($P = 0.00$; **Figure 1**). Ratios of lathosterol to cholesterol (mmol/mol cholesterol) were 1.41 (1.06–2.33) during the control period, 1.80 (0.87–3.09) in the soy sterol ester period, and 1.76 (1.17–2.69) in the β-sitostanol ester period. The ratio of lathosterol to cholesterol was not significantly different between the intervention periods and the control period.

Data on sterol excretion are shown in **Table 2**. Cholesterol and bile acid excretion were not significantly different between periods. Total plant sterol excretion, ie, the sum of brassicasterol, β-sitosterol, β-sitostanol, campesterol, campestanol, stigmasterol, and avenasterol, was 205 mg/d (186–256 mg/d) in the control period, 1447 mg/d (1250–1522 mg/d) in the soy sterol ester period, and 1592 mg/d (1484–1783 mg/d) in the β-sitostanol ester period. The median total recoveries corresponded to 88% with the soy sterol ester and 94% with the β-sitostanol ester.

DISCUSSION

To our knowledge, this is one of the first comparisons of the effects of plant sterols with unsaturated or saturated esterified structures on cholesterol absorption. Our results contribute to the ongoing plant sterol debate, which has centered mostly on the hypothesized stronger effects on cholesterol absorption of saturated plant sterols than of unsaturated plant sterols. Our findings contradict the suggestion that saturated plant sterols, especially β-sitostanol, are more potent inhibitors of cholesterol absorption than unsaturated plant sterols (14). In fact, we found that unsaturated soy sterol esters inhibited cholesterol absorption as efficiently as β-sitostanol. Free plant sterols, however, have been shown to differ in their ability to reduce cholesterol absorption (3) and to lower serum cholesterol concentrations (9). A comparison of free β-sitosterol with free β-sitostanol showed that the saturated plant sterol decreased cholesterol absorption more efficiently than the unsaturated one (3). A comparison of the result from that single intubation study with the results of the present ileostomy study suggests that esterification makes the unsaturated soy sterols comparable to β-sitostanol in this respect.

An earlier comparison of free β-sitosterol with β-sitosteryl oleate in humans showed that the free plant sterol decreased cholesterol absorption more efficiently than the ester during a breakfast meal with a high cholesterol content (30). In this study,

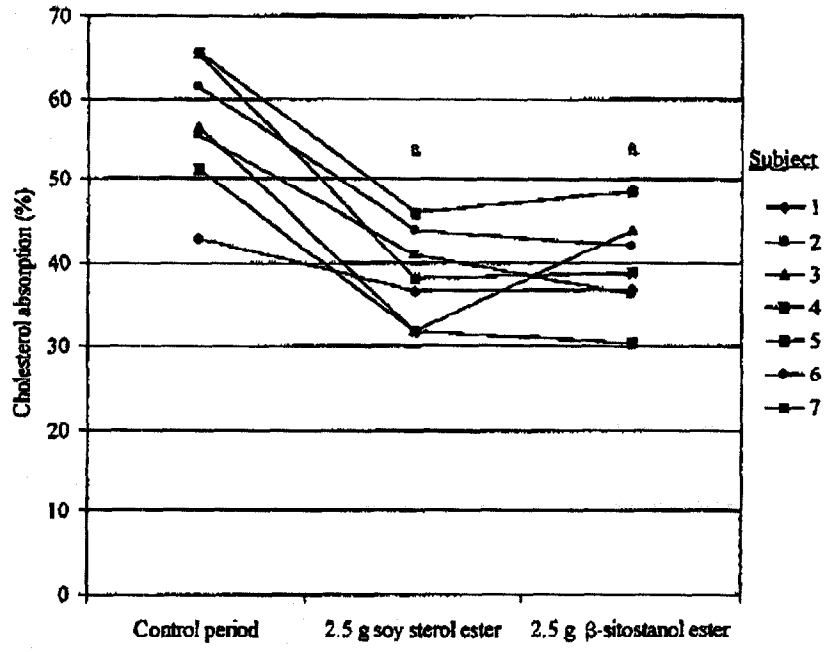

FIGURE 1. Individual responses in cholesterol absorption to plant sterol intervention. Cholesterol absorption was calculated based on the recovered amounts of [β-4-^{14}C]sitosterol and [1α, 2α-n-^{3}H]cholesterol in ileostomy effluents. [a]Significantly different from the control period, $P < 0.05$ (ANOVA and Dunnett test with Bonferroni adjustment).

however, the plant sterols were added to the meal differently: the free form was mixed into the breakfast omelet and the esterified form was blended with the frying fat. To what extent this affected the inhibition of cholesterol absorption is unknown. The difference found between the free and esterified forms may have been due to an incomplete hydrolyzation of the plant sterol esters in the gut, because it is the sterol monohydrate that affects micellar binding (31). Still, it is important to emphasize that in the present ileostomy study, the different plant sterol esters were consumed in exactly the same way.

An important finding supporting the results of the present study was described recently by Weststrate and Meijer (12), who showed that soy sterol esters and β-sitostanol ester result in a similar hypocholesterolemic response. The source of the esters used by Weststrate and Meijer was fortified margarines. Note, however, that the incorporation of plant sterol esters into margarines changes the physicochemical environment of the plant sterol ester. In the present study, the plant sterol esters were not incorporated into the butter, which may explain the different effect on cholesterol absorption from that of the fortified products. However, as in the study by Weststrate and Meijer, the effects of soy sterol esters and β-sitostanol ester seemed to also be comparable when not blended with fat.

In vegetable oils, plant sterols exist in both free and esterified forms. This aspect of plant sterols was addressed in a study comparing 3 dietary regimens: *1*) corn oil with a naturally high plant sterol concentration, *2*) olive oil with a naturally low concentration of plant sterols, and *3*) olive oil enriched in free plant sterols (32). Addition of the mixture of free plant sterols to the olive oil in the third regimen did not result in the same serum cholesterol–lowering effect as produced by the corn oil regimen, even though the third regimen contained 2-fold more plant sterols than the first. Serum plant sterol concentrations in the 3 periods were not significantly different, despite the differing dietary intakes of plant sterols. Plant sterols are absorbed after micellar incorporation in the human small bowel (33) and the lack of increase in serum concentrations indicates that the plant sterols had not been dissolved in the micelles. To make the addition of plant sterols comparable, they should have been added as a mix of free and esterified plant sterols, as in the natural oils. This may explain the unexpected response in serum cholesterol elicited by the third regimen.

TABLE 2
Sterol composition of ileostomy excreta during the 3 study periods.[1]

Sterol	Basal diet	Basal diet + 2.5 g soy sterol ester	Basal diet + 2.5 g β-sitostanol ester
		mg/24 h	
Cholesterol	1066 (776–1277)	1216 (1074–1288)	1204 (1018–1323)
Cholic acid	320 (80–646)	230 (56–502)	297 (58–560)
Chenodeoxycholic acid	232 (42–310)	136 (36–263)	182 (44–325)

[1]Median; range in parentheses. Changes in cholesterol absorption were tested by using ANOVA and the Dunnett test. There were no significant differences between any of the study periods.

The present study showed that soy sterol ester and β-sitostanol ester inhibited cholesterol absorption, although there were no major changes in sterol excretion. The 4 possible mechanisms for serum cholesterol lowering, as suggested by Spritz et al (34), are *1*) decreased cholesterol synthesis; *2*) increased excretion of cholesterol, bile acids, or both; *3*) shift of plasma cholesterol to other tissues; or *4*) a combination of the 3 prior mechanisms. The main explanation for the serum cholesterol–lowering effect of plant sterols seems to be an inhibition of cholesterol absorption, which thereby increases cholesterol excretion. The lack of a significant increase in cholesterol excretion in the present study may be explained by the low power of the study as a result of the low number of subjects.

An increase in bile acid excretion, as suggested by Becker et al (9), is not supported by the results of the present study. Bile acid excretion was slightly decreased with soy sterol esters, although not significantly, and not changed at all with β-sitostanol ester. Earlier studies of plant sterols in humans and animals also showed conflicting results for bile acid excretion, probably because of the small group sizes and variations in fecal output (7, 9, 14, 35–39). Moreover, sterol excretion in response to a plant sterol intervention may depend on the plant sterol and cholesterol dose (37). Studies have been performed with ratios of plant sterol to cholesterol of 2–250; furthermore, some authors have given no description of cholesterol intake. Different plant sterols may also induce various effects on bile acid excretion. A study of pure stigmasterol showed increased bile acid excretion (38). In a clinical study of children with familial hypercholesterolemia, the addition to the diet of saturated β-sitostanol increased bile acid excretion significantly, whereas β-sitosterol had no significant effect on bile acid excretion (9). The wide range of ratios of plant sterol to cholesterol used in supplementation studies and the lack of systematic testing of different isolated structures makes it difficult to evaluate the effects of plant sterols on bile acid excretion.

Because lathosterol-to-cholesterol ratios reflect hepatic cholesterol synthesis (40), increased ratios found after the addition of plant sterol esters seem to reflect increased hepatic synthesis as a result of the reduced uptake of cholesterol by the gut. Six-week regimens with intakes of β-sitostanol ester corresponding to 3.4 g and <1 g β-sitostanol/d were shown to result in increased lathosterol-to-cholesterol ratios (14, 40). Increased hepatic cholesterol synthesis does not seem to be sufficient to balance the inhibition of cholesterol absorption. The lack of statistical increase in the present study was probably the result of the small number of subjects.

In conclusion, we showed that esterified soy sterols and β-sitostanol inhibited cholesterol absorption equally, despite the different structures of the plant sterols. Thus, the efficiency of plant sterols is not only attributable to their chemical structures.

We thank Ingmar Wester of The Raisio Group, Raisio, Finland, for providing the plant sterol esters; Birgitta Frones for her excellent assistance during the study; and Susan Andersson for her language corrections.

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Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters

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Abstract It has been suggested that phytosterol and phytostanol esters possess similar cholesterol-lowering properties, however, whether mechanisms responsible are identical has not been addressed. To address this question, cholesterol plasma levels, absorption, biosynthesis, and turnover were measured in 15 hypercholesterolemic subjects consuming prepared diets each over 21 d using a crossover design. Diets contained either *i*) margarine (M), *ii*) margarine with phytosterol esters (MSE) (1.84 g/d), or *iii*) margarine with phytostanol esters (MSA) (1.84 g/d). Cholesterol absorption was measured using the ratio of [^{13}C]cholesterol$_{oral}$:D$_7$-cholesterol$_{IV}$; biosynthesis using D incorporation from D_2O and turnover by D$_7$-cholesterol$_{IV}$ decay rates. Plasma total cholesterol level at d 21/22 was lower ($P < 0.05$) for MSE (13.4%) but not MSA (10.2%) versus M (6.0%) diets. Plasma low density lipoprotein-cholesterol (LDL-C) mean reductions at d 21/22 were larger ($P < 0.05$) for MSE (12.9%) and MSA (7.9%) compared with M (3.9%). Plasma TG and high density lipoprotein-cholesterol (HDL-C) levels did not differ across diets. Cholesterol absorption was reduced ($P < 0.05$) 36.2 and 25.9% at d 21 for MSE and MSA versus M, while cholesterol biosynthesis was reciprocally increased ($P < 0.05$) 53.3 and 37.8% for MSE and MSA versus M, respectively. Cholesterol turnover was not influenced by diet. These data indicate that plant sterol and stanol esters differentially lower circulating total and LDL cholesterol levels by suppression of cholesterol absorption in hypercholesterolemic subjects.—Jones, P. J., M. Raeini-Sarjaz, F. Y. Ntanios, C. A. Vanstone, J. Y. Feng, and W. E. Parsons. **Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters.** *J. Lipid Res.* 2000. 41: 697–705.

Supplementary key words phytosterols • phytostanols • cholesterol • absorption • synthesis

Phytosterols, including β-sitosterol, campesterol, and stigmasterol, naturally occur in plants in both free and esterified form, as well as conjugated as glycosides (1). Saturation of phytosterols at the 5-alpha position form compounds including sitostanol and campestanol. For several decades it has been appreciated that consumption of plant sterols and stanols led to favorable shifts in circulating lipid levels (1–13). In general terms, plant sterol and stanol consumption in human subjects under a range of experimental circumstances reduces plasma total and low density lipoprotein cholesterol (LDL-C) concentrations within the range of 0.5–26% and 2–33%, respectively (ref. 14 for review). Indeed, addition of plant sterols and stanols to foods for the purpose of lowering plasma cholesterol concentrations presently reflects a major development in the functional foods area in Europe and North America.

There remains controversy, however, over the relative cholesterol-reducing efficacy of plant sterols versus stanols. A recent finding that esters of unsaturated β-sitosterol and campesterol produce the same cholesterol-lowering efficacy as esters of sitostanol and campestanol, when added to margarines (10), is somewhat inconsistent with previous animal data (4, 5, 7, 11). This earlier data from animals suggest that efficacy of cholesterol-lowering action increases with the extent of hydrogenation of the phytosterol mixture.

For this reason, the aim of the present investigation was to re-examine whether margarines containing esters of unsaturated plant sterols possess the same efficacy as those containing esters of saturated plant sterols in the modification of circulating lipoprotein cholesterol levels in hypercholesterolemic subjects. The secondary objective was to determine whether alterations in cholesterol absorption, turnover, or synthesis could account for any relative actions of esters of unsaturated versus saturated plant sterols on circulatory lipid levels. To achieve this aim, the current study examined the effect of feeding esterified plant sterols and stanols in margarine mixtures on sterol metabolism in hyperlipidemic males consuming prepared,

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Abbreviations: LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; M, margarine diet; MSE, margarine + phytosterol esters diet; MSA, margarine + phytostanol esters diet; TC, total cholesterol; TG, triglyceride; RBC, red blood cells; FSR, fractional synthesis rate.

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fixed foods diets. The null hypothesis was that feeding the margarine alone, versus that with added plant sterol or stanol esters, to mildly hyperlipidemic subjects for 3 weeks would not influence their plasma lipid profiles, cholesterol absorption, turnover, or synthesis, nor plasma phytosterol levels.

METHODS

Human subjects

Hyperlipidemic males (37 to 61 yr) participated in the study. Subjects were screened for plasma total cholesterol (TC) and triglyceride (TG) levels. Criteria for acceptance were fasting plasma TC concentrations in the range of 6.0 to 10.0 mmol/L and TG less than 3.0 mmol/L. Subjects were screened for familial hypercholesterolemia using a family history questionnaire. Individuals reporting a personal history of diabetes, heart disease, or hypothyroidism, or who had been using drug therapy for hypercholesterolemia at any time during the 2 months prior to the start of the study, were excluded. The experimental protocol was approved by the Ethics Committee of the Faculty of Agriculture and Environmental Sciences for the School of Dietetics and Human Nutrition at McGill University. All subjects received a thorough explanation of the protocol and were given the opportunity to discuss any queries prior to signing a consent form.

Protocol and diet

Using a randomized crossover double-blind design, three coded margarine mixtures were assigned to the three dietary treatments; one contained margarine alone, a second contained margarine with 8% (wt/wt based on free sterol content) plant sterol esters, and a third contained 8% (wt/wt based on free stanol content) plant stanol esters. Plant sterols were derived from vegetable oil and esterified with fatty acids obtained from rapeseed oil. Similarly, plant stanols were derived from vegetable oil, hydrogenated, then esterified with fatty acids from rapeseed oil. Fatty acids esterified to sterol and stanol esters included C18:1n-9 (58.6%), C18:2n-6 (18.9%), and C18:3n-3 (9.9%). Sterol and stanol esters were dissolved in margarine during their formulation. Margarine fatty acid and phytosterol composition is given in **Table 1**. To ensure that the crossover design was balanced, subjects were randomly assigned to one of six predetermined Latin squares, where each square possessed 3 sequenced phases and 3 subjects.

Subjects consumed a fixed intake North American solid foods diet during each of the three treatments. Each phase consisted of a 21-day feeding period followed by a 5-week washout. The diet was formulated to meet Canadian Recommended Nutrient Intakes. Dietary protein, carbohydrate, and fat made up 15, 50, and 35% of ingested energy, respectively. Dietary fat was comprised of 15, 10, and 10% of energy as monounsaturated, saturated, and polyunsaturated fats, respectively, using a blend of olive oil, butter, and a sunflower oil-based margarine. The diet was designed using a 3-day rotating meal cycle to provide variety over feeding periods. Meals were prepared in the Mary Emily Clinical Nutrition Research Unit metabolic kitchen. Subjects were required to consume a minimum of 2 meals per day, including breakfast, at the Nutrition Research Unit under the supervision of the unit's staff. Subjects were repeatedly instructed not to consume any food or beverages other than those provided by the Nutrition Research Unit.

The amount of food consumed by each subject was formulated to maintain individual weight balance, using a predictive equation based on each subject's weight, height, age, and activity level (15). Weight changes were monitored daily and food amounts were adjusted accordingly where necessary.

Margarine mixtures were incorporated into respective diets at a mean level of 23 g margarine per day, corresponding to 1.84 g free sterols or stanols, per day. Daily margarine doses were added to each meal divided into equal portions.

Blood samples were collected from subjects before breakfast on days 0, 8, 15, 18, 19, 20, 21, and 22 of each feeding period. On days 0 and 21 of each phase an additional blood sample was drawn to perform complete blood analyses to monitor the

TABLE 1. Plant sterol and fatty acid composition of spreads

	Sitostanol Ester Treatment		β-sitosterol Ester Treatment		Control	
Composition	mg kg^{-1}	% (w/w)	mg kg^{-1}	% (w/w)	mg kg^{-1}	% (w/w)
Total plant sterols						
Cholesterol	202	0.26	470	0.56	30	1.2
Brassicasterol	173	0.23	1251	1.49	240	11.3
Campesterol	1646	2.14	21611	25.80	610	28.9
Campestanol	21304	27.79	770	0.92	40	2.1
Stigmasterol	743	0.97	16106	19.23	40	2
β-Sitosterol	3868	5.04	38402	45.85	980	46.9
Sitostanol	46770	61.02	1610	1.92	20	1.1
d5-Avenasterol	133	0.17	838	1.00	60	2.9
Other sterol	1700	2.22	2700	3.22	80	3.6
Total	76639	100	83757	100	2100	100
Total fat (as glycerides)	g kg^{-1}	% (w/w)	g kg^{-1}	% (w/w)	g kg^{-1}	% (w/w)
Lauric (C12:0)	7.0	1.8	10	2.2	10	2.3
Myristic (C14:0)	4.0	1.0	5	1.2	5	1.2
Palmitic (C16:0)	43	10.5	51	11.7	51	11.8
Stearic (C18:0)	13	3.2	14	3.2	13	3.1
Oleic (C18:1 9c)	166	40.5	174	39.7	181	41.8
Linoleic (C18:2 9c,12c)	137	33.4	143	32.6	130	30.0
Linolenic (C18:3 9c,12c, 15c)	23	5.6	24	5.5	25	5.9
Other fatty acids	17	4.2	17	3.9	17	4.0
Total	411	100	439	100	433	100

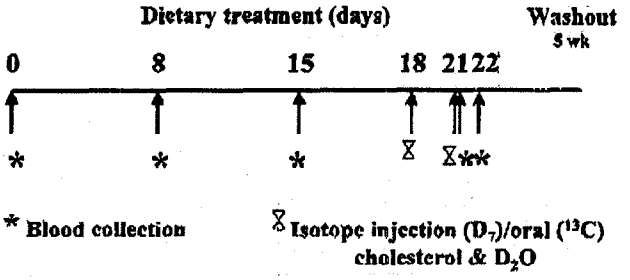

Fig. 1. Protocol time-line used in study.

health of the subjects. Subjects also underwent complete physical examinations and urinalyses at the beginning and end of each dietary treatment.

Ninety-six hours before the end of the trial on day 18, subjects were intravenously (iv) injected with 15 mg D_7-cholesterol and simultaneously ingested 90 mg [3,4-^{13}C]-cholesterol. Five subjects during the first phase did not receive D_7-cholesterol. In these cases, the average enrichment from the other two phases was calculated and utilized as a surrogate for the true D_7-cholesterol enrichment. The D_7-cholesterol isotope was prepared for injection by dissolving it in ethanol at a concentration of 5 mg/ml under sterile conditions. The isotope/ethanol mixture was then added drop-wise to Intralipid™ for a total injectable volume of 9 ml. Blood samples were taken at baseline, 6 h, and 12 h on day 18, as well as fasting samples on days 19, 20, and 21 to monitor isotopic enrichment/decay levels. On day 21 of each feeding period, approximately 25 ml of deuterium oxide was given orally to each subject. The change in deuterium enrichment within red blood cell (RBC) free cholesterol was determined as an index of synthesis over days 21 and 22 (72 and 96 h after initial isotope administration). The time line for the protocol used is provided in Fig. 1.

Lipoprotein lipid analyses

Blood was centrifuged for 15 min at 1,500 rpm within 30 min of phlebotomy to separate plasma from RBCs. Plasma and RBCs were immediately stored at −80°C until further analysis. Plasma TC, high density lipoprotein cholesterol (HDL-C), and TG concentrations were analyzed in quadruplicate with enzymatic kits, standardized reagents, and standards using a VP Autoanalyzer (Abbott Laboratories, North Chicago, IL). The equation of Friedewald, Levy, and Fredrickson (16) was used to calculate LDL-C concentrations.

Determination of cholesterol absorption

Cholesterol absorption was determined using the dual stable isotope ratio technique of Bosner et al. (17). Free cholesterol extracted from RBCs was used to determine ^{13}C- and D_7-cholesterol enrichment. Red cells contain almost exclusively free cholesterol designated as part of the rapid changeover pool. Briefly, lipid was extracted from RBC in duplicate using a modified Folch, Lees, and Sloane Stanley extraction procedure (18). Thin-layer chromatography (20 × 20 cm, 250 μ, Scientific Adsorbents Inc., Atlanta, GA) was used to separate free cholesterol from cholesteryl ester. The free cholesterol band was scraped from the silica gel plate and saponified with 0.5 M methanolic KOH to eliminate any fatty acid contaminants. Free cholesterol extracts were dried under nitrogen and transferred into 18-cm sealed combustion tubes (Vycor, Corning Glass Works, Corning, NY). Cupric oxide (0.5 g) and a 2-cm length of silver wire were added and tubes were sealed at less than 20 mtorr pressure. Cholesterol samples were then combusted to ^{13}C-enriched CO_2 and D-enriched water for 4 h at 520°C. The generated CO_2 was transferred under vacuum into Vycor tubes for measurement of ^{13}C enrichment, while D-enriched water was vacuum-distilled into sealed tubes containing 60 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington, IN). Tubes containing water and zinc were reduced to D-enriched hydrogen gas at 520°C for 30 min.

Nuclear magnetic resonance was used to verify that the isotopic enrichments of the starting materials [3,4]-^{13}C-cholesterol and D_7-cholesterol (CDN Isotopes, Point Claire, Quebec) were greater than 99 atom percent excess. The ^{13}C enrichments of free cholesterol were measured by differential isotope ratio mass spectrometry (IRMS) using an automated dual inlet system (SIRA 12, Isomass, Cheshire, UK). Enrichments were expressed relative to Pee Dee Belemnite (PDB) limestone standard of the National Bureau of Standards (NBS). Linearity and gain of response of the SIRA IRMS instrument were assessed using a reference tank CO_2 and NBS standards of known isotopic enrichment. The D enrichments of free cholesterol were measured by differential IRMS using a manually operated dual inlet system with electrical H^{3+} compensation (VG Isomass 903D, Cheshire, UK). For deuterium, enrichments were expressed relative to Standard Mean Ocean Water (SMOW) and a series of NBS standards of known enrichment were analyzed concurrently on each day of measurement to correct for any variations in linearity of gain of response of the 903D IRMS. Precision of measurement expressed as coefficient of variation for replicate ^{13}C and D enrichment analyses was 0.08 and 2.8 *del* (parts per thousand relative to PDB and SMOW standards), respectively. The ^{13}C and D enrichments in 48 and 72 h RBC free cholesterol relative to baseline (t = 0) samples were utilized to calculate the percent cholesterol absorption using the ratio of orally ingested ^{13}C- to intravenously administered D-cholesterol as described by Bosner et al. (17) where:

$$\text{absorption (pool/pool)} = \frac{del^{13}\text{C} \times 7 \times \text{iv dose of D-cholesterol(mg)} \times 0.0112}{del\text{D} \times 2 \times \text{i.g. dose of } ^{13}\text{C-cholesterol(mg)} \times 0.000155} \quad \textit{Eq. 1)}$$

where *del* for ^{13}C and D is the difference between the enriched sample at 48 or 72 h and the baseline abundance (at t = 0) in parts per thousand relative to PDB and SMOW standards, respectively, the factor 7/2 reflects the ratio of labeled atoms per mg of dose, and the constants 0.0112 and 0.000155 represent factors converting the *del* to equivalent atom percent excess for the PDB and SMOW scales, respectively.

Determination of cholesterol biosynthesis and turnover

Cholesterol biosynthesis was determined as the rate of incorporation of deuterium from body water into RBC membrane free cholesterol over the period between 72 and 96 h at the end of each treatment as described (19). Fractional synthesis rate (FSR) represents that fraction of the cholesterol pool that is synthesized in 24 h and is calculated as per the equation (20, 21):

$$\text{FSR (pools/day)} = (del_{\text{cholesterol}}/del_{\text{plasma}}) \times 0.478 \quad \textit{Eq. 2)}$$

where *del* for D enrichment in cholesterol is the difference between enriched free cholesterol and plasma water at 72 and 96 h in parts per thousand relative to a SMOW standard. The factor 0.478 reflects the ratio of labeled H atoms replaced by D ($^{22}/_{46}$) during in vivo biosynthesis (19).

Turnover rates for RBC free cholesterol were determined from the D_7-cholesterol enrichment decay curve over 24 to 72 h. Exponential curves were fitted to 24, 48, and 72 h RBC D_7 cho-

lesterol enrichments after subtraction of baseline D abundance at 0 h.

Determination of plasma plant sterol levels

Plant sterols were measured by gas–liquid chromatography (GLC) (HP 5890 Series II, Palo Alto, CA) equipped with flame ionization detection and auto-injector system as described (11, 13). A 30-m SAC-5 column (Sigma-Aldrich Canada Ltd., Oakville, Ont.) was used. Briefly, an internal standard, 5 alpha-cholestane, was added to each plasma sample. Samples were saponified and sterols were extracted, re-suspended in chloroform, and injected into the GLC. The column temperature was 285°C. Isothermal running conditions were maintained for 42 min. The injector and detector were set at 300°C and 310°C, respectively. The carrier gas (helium) flow rate was 1.2 ml/min with the inlet splitter set at 100:1. Individual plant sterol and stanols were identified using authentic standards (Sigma-Aldrich Canada Ltd., Oakville, Ont). Internal standards were used to calculate detector response factors. Margarine mixtures were also analyzed by GLC to assess sterol and stanol, as well as fatty acid, content using similar procedures.

Analysis of data

Data were expressed as the mean ± standard error mean (SEM). For lipoprotein cholesterol, TG, and phytosterol levels, data at commencement and end of each dietary period were compared using a crossover repeated measures ANOVA model to identify time and treatment effects and their interactions. Endpoints for lipid level data were taken as averages of day 21 and 22 values. For cholesterol absorption, synthesis, and turnover, data at the end of each dietary period were compared using a crossover ANOVA model to identify treatment effects. When treatment effects were identified as significant, Duncan's post-hoc tests were utilized to identify significant effects of diet at particular timepoints. Separate comparisons were performed on endpoints for circulating lipid levels using ANOVA followed by Students' paired t-tests with Bonferroni adjustment to control the overall alpha level. Tests for associations between variables were also performed using Pearson Correlation Coefficient analyses. Specifically, associations were investigated between circulating sterol levels at the end of each treatment and cholesterol absorption, cholesterol biosynthesis, and plant sterol levels. The data were analyzed using Proc-General Linear Model SAS (version 6.12) software.

RESULTS

Subject compliance and drop-out rate

Eighteen subjects commenced the study protocol, with sixteen subjects completing all three treatments. However, data from only 15 subjects were included in final analyses because two subjects left the study at the end of the first phase due to personal reasons and a third was terminated due to poor compliance.

Subject blood and urine parameters and demographic response to treatment

Complete blood counts (CBC), biochemistry (SMAC), and urinalysis results during the three phases of the trial remained within normal ranges. Regular physical exams revealed no suggestion of any clinical irregularities. No significant mean group weight changes occurred across any of the three treatments. Subjects tolerated the diet without any reported adverse effects, reporting no abnormal or atypical smell, taste, color, or mouth-feel effects across treatments. Subjects were unable to distinguish between dietary treatments.

Plasma lipid profile in response to treatment

Plasma TC concentrations measured across all treatments of the feeding trial showed large between-subject variation (**Table 2**), while the time by diet interaction was

TABLE 2. Plasma lipid levels at days 0 and mean of days 21 and 22 of each dietary period

Lipid	β-Sitosterol Ester	Sitostanol Ester	Control
		mmol L^{-1}	
Total cholesterol			
Day 0	6.39 ± 0.18	6.37 ± 0.18	6.47 ± 0.22
Day 21/22	5.49 ± 0.15^{**b}	5.71 ± 0.18^{**ab}	6.04 ± 0.18^{*a}
% Change	−13.4 ± 2.6^{b}	−10.2 ± 2.1^{ab}	−6.0 ± 2.4^{a}
% Relative to control	−9.1	−5.5	
Low density lipoprotein			
Day 0	4.29 ± 0.25	4.35 ± 0.23	4.46 ± 0.25
Day 21/22	3.66 ± 0.15^{**c}	3.95 ± 0.19^{*b}	4.22 ± 0.18^{a}
% Change	−12.9 ± 3.0^{b}	−7.9 ± 3.7^{ab}	−3.9 ± 3.1^{a}
% Relative to control	−13.2	−6.4	
Triglyceride			
Day 0	2.52 ± 0.21	2.39 ± 0.20	2.24 ± 0.21
Day 21/22	1.98 ± 0.21*	1.86 ± 0.16**	1.93 ± 0.15
% Change	−18.9 ± 8.3	−17.4 ± 5.6	−5.7 ± 9.3
% Relative to control	1.0	0.9	
High density lipoprotein			
Day 0	0.99 ± 0.06	0.92 ± 0.05	0.98 ± 0.06
Day 21/22	0.93 ± 0.06	0.93 ± 0.05	0.93 ± 0.06
% Change	−5.6 ± 2.3	0.8 ± 2.7	−4.5 ± 3.2
% Relative to control	0.0	0.0	

Values are expressed as mmol/L ± SEM. Values carrying different superscript letters indicate that there is a significant difference between mixtures ($P < 0.05$). Percent change is based on individual data. Percent change relative to control is based on the mean for days 21 and 22.

*$P < 0.05$; ** $P < 0.01$: significant differences within each diet (between day 0 and mean of days 21 and 22).

marginally significant ($P = 0.09$). Main effects of time and diet ($P < 0.0001$ and 0.05, respectively) were significant for TC levels. Mean reductions in total cholesterol over days 21 and 22, relative to day 0, were 13.4 ± 2.6, 10.2 ± 2.1, and 6.0 ± 2.4% for sterol ester, stanol ester, and control treatments, respectively. When means of TC levels at the end of each phase were compared, control values were higher than those after consumption of the sterol ($P < 0.005$), but not stanol ester-containing diets (**Fig. 2**). The decline in TC levels at the end of sterol and stanol ester phases, relative to that at the end of the control phase, were 9.1 and 5.5%, respectively.

As with TC level, between-individual variation in LDL-C concentration responses were substantial (Table 2). While time-by-diet interactions were not significant, main effects of time ($P = 0.0001$) and diet ($P < 0.01$) for LDL-C levels were observed. Mean reductions in LDL-C levels over days 21 and 22, relative to day 0, were 12.9 ± 3.0, 7.9 ± 3.7, and 3.9 ± 3.1% for sterol ester, stanol ester, and control treatments, respectively. Both the sterol and stanol ester-containing margarines decreased ($P < 0.02$) LDL-C concentration relative to control (**Fig. 3**). Moreover, significant differences were identified among all three treatments at days 21/22, with the lowest levels observed with the sterol ester margarine. The declines in LDL-C levels at the end of the sterol and stanol ester phases, relative to control, were 13.4 and 6.4%, respectively.

TG and HDL-C concentrations are listed in Table 2. There were no significant effects of diet on either TG or HDL-C levels with either of the margarines. However, a significant main effect of time ($P < 0.005$) was found for TG and HDL-C, with levels declining over time in each case. Plant sterol and stanol-induced changes in both TG and HDL-C, relative to changes in control, were minimal (Table 2).

Cholesterol absorption in response to treatment

Cholesterol absorption data, provided as enrichment values for ^{13}C- and D-labeled cholesterol in RBC free cholesterol are presented in **Fig. 4.** At 48 h after isotope administration, mean cholesterol absorption coefficient (cholesterol oral dose/cholesterol iv dose) was lower ($P < 0.005$) after sterol ester and stanol ester feeding (0.387 ± 0.056 and 0.454 ± 0.087 pool/pool, respectively), relative to control feeding (0.664 ± 0.078 pool/pool). There was,

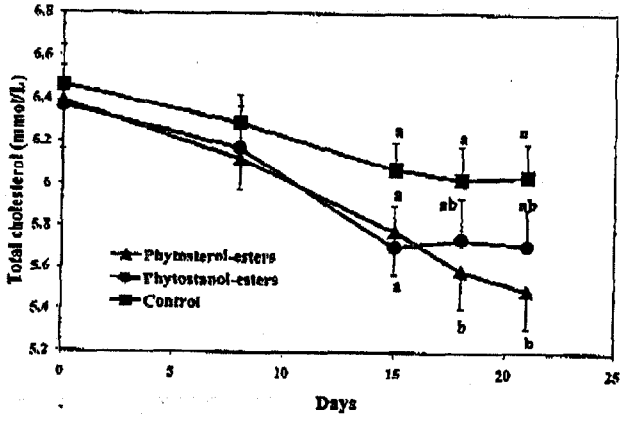

Fig. 2. Plasma total cholesterol levels of subjects over each dietary period. Different letters indicate significant differences ($P < 0.02$) among diets. Endpoint data represent means of values obtained on days 21 and 22. Bars represent SEM (standard error of mean).

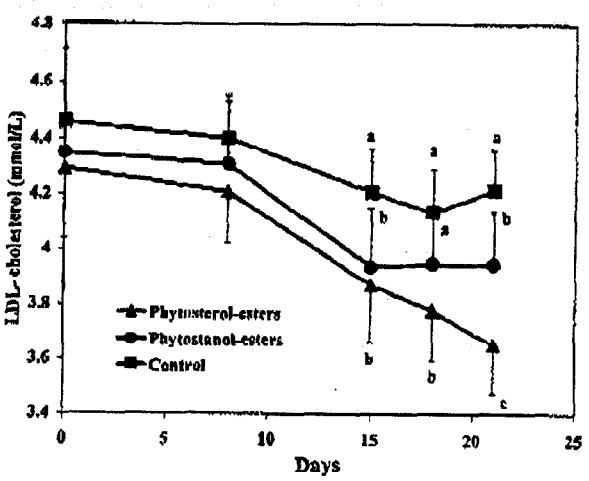

Fig. 3. Plasma low density lipoprotein levels of subjects over each dietary period. Different letters indicate significant differences ($P < 0.02$) among diets. Endpoint data represent means of values obtained on days 21 and 22. Bars represent SEM (standard error of mean).

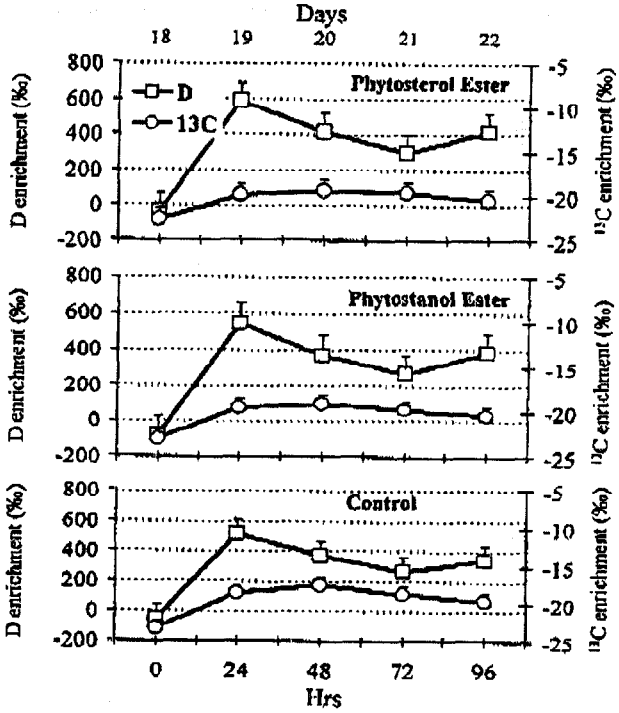

Fig. 4. Enrichment of ^{13}C and D in plasma cholesterol during the final week of each dietary period. Bars represent SEM (standard error of mean).

however, no difference observed in cholesterol absorption between the sterol versus stanol-containing diets. The trend in absorption rate tended to be proportional to the extent of cholesterol-lowering, particularly in the case of LDL-C.

At 72 h, mean cholesterol absorption coefficient was also marginally lower ($P = 0.08$) after sitosterol ester (0.488 ± 0.078 pool/pool), relative to control (0.709 ± 0.092 pool/pool) diets. There was, however, no difference observed in cholesterol absorption between the two diets containing phytosterols, or between the stanol ester (0.550 ± 0.122 pool/pool) and control diets. The trend in absorption here also tended to directly mirror the degree of cholesterol-lowering in that the group with the lowest LDL-C levels in the group displayed the most suppressed cholesterol absorption.

When means of each time-point (i.e., 48 and 72 h) were computed for each subject, cholesterol absorption was lower ($P < 0.01$) after sterol and stanol ester feeding (0.438 ± 0.062 and 0.502 ± 0.103 pool/pool, respectively), relative to control (0.687 ± 0.083 pool/pool) diet. Relative to control, absorption was reduced 36.2 and 25.9% for sterol ester and stanol ester-containing diets, respectively. There was, however, no difference in cholesterol absorption coefficient between the sterol versus stanol-containing diets. At day 21, the group provided with sterol esters displayed both the lowest LDL-C levels and the lowest cholesterol absorption coefficient.

Cholesterol synthesis in response to treatment

Deuterium enrichments in RBC cholesterol after deuterated water during 72–96 h post-infusion are shown in Fig. 4. Fractional synthesis rates were higher ($P < 0.05$) after sterol and stanol ester feeding (0.0535 ± 0.0069 and 0.0481 ± 0.0054 pools/day, respectively), relative to the control diet (0.0349 ± 0.0043 pools/day). Relative to control, synthesis was increased 53.3 and 37.8% for sterol and stanol ester-containing diets, respectively. There was, however, no statistically significant difference observed in synthesis rates between the sterol and stanol-containing diets. The trend in synthesis rate tended to inversely mirror the degree of cholesterol-lowering and cholesterol absorption coefficient. At day 21, the lowest LDL-C levels in the group provided with sterol esters corresponded with a similar trend towards higher cholesterol biosynthesis within the same group.

Cholesterol turnover in response to treatment

Turnover rates were obtained from the cholesterol deuterium enrichment data over 24–72 h (Fig. 4) for treatments containing sterol esters, stanol esters, and control margarines. RBC free cholesterol turnover rates were 0.310 ± 0.16, 0.308 ± 0.14, and 0.293 ± 0.21 pools/day, respectively. There were no significant differences observed between diets.

Plasma plant sterol profile in response to treatment

Plasma phytosterol concentrations and ratios are presented in **Table 3**. Mean plasma concentrations of campesterol and β-sitosterol at day 21 increased ($P < 0.0001$) compared with day 0 with consumption of sterol ester diet by 71.6 and 32.5%, respectively, and were significantly different ($P < 0.05$) from the changes observed with consumption of stanol esters and control diets. The ratio of campesterol:β-sitosterol with sterol ester increased ($P < 0.0001$) over time by 32.7%, which was different ($P < 0.05$) from the changes observed with the stanol ester and control diets. Consumption of the sterol ester-containing diet increased ($P < 0.0001$) both the campesterol:cholesterol and β-sitosterol:cholesterol ratios by 94.2 and 53.5%, respectively, and both ratios differed from those of diets containing stanol esters or the control margarine. Even though consumption of the stanol ester margarine decreased ($P < 0.0001$) the campesterol:cholesterol and β-sitosterol: cholesterol ratios by 19.7 and 13.8%, respectively, neither ratio was significantly different from those after control diets.

Associations between plasma lipid levels and kinetic measurements

For all study subjects, both plasma campesterol ($r = 0.36$, $P < 0.02$) and β-sitosterol ($r = 0.53$, $P < 0.0005$) levels were found to vary directly with plasma TC concentration. Similarly, β-sitosterol ($r = 0.36$, $P < 0.02$) levels were found to vary directly with the level of LDL-C in plasma. Furthermore, whereas cholesterol absorption coefficient was marginally correlated ($P = 0.07$) with TC concentration, there was a stronger association ($r = -0.45$, $P < 0.002$) between cholesterol synthesis and TC concentrations. Moreover, FSR varied inversely with plasma levels of campesterol ($r = -0.32$, $P < 0.05$) and β-sitosterol ($r = -0.45$, $P < 0.005$) in all subjects. When subjects were analyzed by treatment grouping, an association ($r = 0.53$, $P < 0.05$) between TC concentration and cholesterol absorption coefficient was observed for the sterol ester but not for the stanol ester or control diets.

DISCUSSION

This study shows for the first time that, under controlled dietary conditions, plant sterols possess improved efficacy in reducing plasma total and LDL-C concentrations compared with stanol esters at the level of intake presently utilized. This reduction occurred as a result of the ability of sterols and stanols to depress cholesterol absorption while partially de-suppressing cholesterol biosynthesis. The fact that the final circulatory TC level was positively associated with the cholesterol absorption, particularly in the group showing the most pronounced cholesterol lowering, indicates inhibition of absorption as a chief mechanism in the cholesterol-modulating effect of these dietary agents. In addition to sterols and stanols, dietary fatty acid composition may have also been partially responsible for the changes in lipids observed. Levels of all lipids measured, except HDL cholesterol, fell across the control dietary period, likely indicative of the beneficial substitution of unsaturated fat for that typically consumed by subjects.

Although moderate reductions in plasma total and LDL-C concentrations have been demonstrated with the use of plant sterols (8, 9, 14, 22), stanols at similar or lower dosages have been suggested as possessing a greater lipid-

TABLE 3. Plant sterol levels at day 0 and 21 of each dietary period

Phytosterol	β-Sitosterol Ester	Sitostanol Ester	Control
		mmol L^{-1}	
Campesterol			
Day 0	0.0128 ± 0.0015	0.0144 ± 0.0017	0.0124 ± 0.0014
Day 21	0.0209 ± 0.0024**^{a}	0.0099 ± 0.0012*^{b}	0.0119 ± 0.0016^{b}
% Change	71.6 ± 13.1^{a}	−27.9 ± 7.6^{b}	4.1 ± 12.6^{b}
% Relative to control	75.6	−16.8	
		mmol L^{-1}	
β-Sitosterol			
Day 0	0.0086 ± 0.0007	0.0100 ± 0.0009	0.0085 ± 0.0008
Day 21	0.0112 ± 0.0012**^{a}	0.0076 ± 0.0009**^{b}	0.0086 ± 0.0010^{b}
% Change	32.5 ± 10.9^{a}	−22.7 ± 7.6^{b}	7.6 ± 10.9^{a}
% Relative to control	30.2	−11.6	
		mol mol^{-1}	
Campesterol:β-sitosterol			
Day 0	1.446 ± 0.078	1.408 ± 0.088	1.427 ± 0.046
Day 21	1.857 ± 0.049**^{a}	1.308 ± 0.055^{b}	1.388 ± 0.065^{b}
% Change	32.7 ± 6.8^{a}	−4.7 ± 4.2^{b}	−1.6 ± 5.1^{b}
% Relative to control	33.8	−5.8	
		mmol mol^{-1}	
Campesterol:total cholesterol			
Day 0	0.199 ± 0.022	0.223 ± 0.024	0.185 ± 0.017
Day 21	0.371 ± 0.037**^{a}	0.168 ± 0.018**^{b}	0.192 ± 0.021^{b}
% Change	94.2 ± 12.3^{a}	−19.7 ± 8.9^{b}	13.5 ± 14.6^{b}
% Relative to control	93.2	−11.9	
		mmol mol^{-1}	
β-Sitosterol:total cholesterol			
Day 0	0.132 ± 0.010^{ab}	0.157 ± 0.013^{a}	0.128 ± 0.011^{b}
Day 21	0.198 ± 0.017**^{a}	0.128 ± 0.013*^{b}	0.142 ± 0.014^{b}
% Change	53.5 ± 11.3^{a}	−13.8 ± 9.6^{a}	19.4 ± 13.7^{b}
% Relative to control	39.4	−9.8	

Values are expressed ± SEM. Different superscript letters indicate significant differences ($P < 0.05$) between diets. Percent change is based on individual data; percent change relative to control diet for day 21.
* $P < 0.05$; ** $P < 0.01$: significant difference within each diet (between day 0 and day 21).

reducing capacity (23–29). However, only one previous study performed a systematic cross-comparison of sterol and stanol esters in adults (10). Here, consumption of esterified sterol and stanol mixtures resulted in reductions in plasma TC concentrations, which were for the greater part indistinguishable (10). Differences between study design including phytosterol dosage or formulation, or dietary composition may be responsible for the variable results observed between the present and previous (10) studies.

Major sub-objectives of the present research were to assess mechanisms of action through which these plant materials act. To meet these objectives, we utilized a novel combination of techniques for measurement of cholesterol absorption, biosynthesis, and turnover using three isotopes provided simultaneously, cholesterol labeled with ^{13}C and D and deuterated water. Each technique possesses strengths and drawbacks.

The approach used to measure cholesterol absorption presently is that of the accepted procedure described by Bosner et al. (17), which assesses the ratio in the plasma pool of labeled cholesterol given orally versus that provided intravenously. However, Bosner et al. (17) measured enrichments with selected ion monitoring mass spectrometry; our study is the first to use more sensitive isotope ratio mass spectrometry, together with ^{13}C and D labeled cholesterol tracers. We (11) and others (30) have previously utilized the ratio of ^{18}O to ^{13}C cholesterol to determine its coefficient of absorption in animals and in humans. However, both current problems of ^{18}O isotope availability and the corrections needed when ^{18}O-labeled cholesterol is combusted with copper oxide resulted in the substitution of polydeuterated cholesterol in the present experiment.

The dual isotope absorption measurement method uses intravenously administered labeled cholesterol as a tracer to monitor of exit rate of both body and dietary cholesterol from the rapid pool relative to orally administered cholesterol labeled with a second tracer to monitor the rate of intestinal absorption. This ratio allows for correction in turnover rate possibly affected by diet or physiological state. Presently we observed that turnover rates of cholesterol were 25–30% per day and did not vary across dietary treatments. However, when the coefficient of absorption was calculated using equation 1, it was demonstrated that absorption rate was reduced significantly with the consumption of the sterol ester-containing diet, and reduced at a level that approached statistical significance with the consumption of the stanol ester diet. The levels

of reduction of cholesterol absorption with sterol and stanol ester diets, relative to control (36.3 and 26.9%, respectively), are not substantially different from those reported in animals for stanol esters (36.1%) at comparable doses (11). In humans using serum cholestanol/cholesterol ratios as an index of cholesterol absorption, stanol ester mixtures provided at approximately 3 g/d for 6 weeks resulted in a decline in absorption of 6 to 21% relative to control diets (31).

The use of deuterium as a marker for cholesterol biosynthesis is based on the methodology developed in animals (19) using radioactive tritiated water. Early work (32) has shown that the equilibration of deuterated cholesterol across plasma and red cell sterol pools is extremely rapid, but that attaining a plateau of enrichment is not achieved even after 100 days of constant deuterium enrichment of body water. These data, taken together with our results demonstrating that deuterium uptake is largely linear over 48 h (20), allow a monoexponential linear model to be fit to the deuterated cholesterol enrichment curve. Application of this model facilitates the interpretation of the deuterium incorporation results.

Although the deuterium uptake methodology has been validated against cholesterol balance (21), plasma mevalonic acid levels (33), and more recently mass isotopomer distribution analysis (M. F. Di Buono, P. J. H. Jones, L. Wykes, and L. Baumier, unpublished results), the technique has not been previously applied in a context where baseline deuterium levels were changing, as in the present experiment. The present application of the method used a modified approach, assessing the baseline enrichment of deuterium at 72 h. It is evident from the 24 and 48 h D enrichment profiles (Fig. 4) that the 72 h D enrichment values likely overestimated the true baseline at 96 h, as the D enrichment continued to decrease over 72 to 96 h. Indeed, mean FSR values were lower than those previously observed in other dietary studies (20, 21, 33). It was contemplated that extrapolation of the true 96 h baseline from the three previous time-points might be a possible means of obtaining a more accurate value for FSR. However, the imprecision created in extrapolating cholesterol deuterium incorporation measure is considered to outweigh any systematic underestimation of FSR by using the true 72 h accessible values. The approach taken did permit almost simultaneous measurement of cholesterol absorption, synthesis, and turnover, as well as circulating lipoprotein cholesterol levels, during the period of investigation. Moreover, demonstration of enhanced cholesterol biosynthesis in response to addition of dietary phytosterols confirms the results of previous reports (34) which identify this compensatory mechanism as a part of the metabolic response to the reduction in cholesterol absorption. The increase in synthesis with the sterol and stanol ester-containing diets seen presently is consistent with previous human (34) and animal (11) data.

Concurrent with measurement of cholesterol synthesis and absorption, changes in plant sterol levels in plasma were also detected. Absolute levels of circulatory β-sitosterol and campesterol found in the present study were not dissimilar to those seen previously (13, 14). As previously, we were unable to detect sitostanol in plasma in any group, although this component has been identified in previous work in animals (27). Data in the present experiment show that changes in levels of campesterol and β-sitosterol were diet-dependent. Particularly, 71.6% and 32.5% increases in plasma campesterol and β-sitosterol levels, respectively, were observed during consumption of the sterol ester but not with the consumption of the other diets. These findings indicate that the non-hydrogenated plant sterols are absorbed to a certain extent. The lower reported absorption efficiency of stanols (14) is likely responsible for the failure to observe any increase in the plasma level of this compound after consumption of a stanol ester-enriched diet for 3 weeks. Indeed, a decrease in both β-sitosterol and campesterol levels was noted in subjects consuming stanol esters. The potential effects of changing circulating plant sterol levels after consumption of phytosterols are not known. It has been suggested that individuals heterozygous for the disorder phytosterolemia, a rare genetic inborn disease, absorb more β-sitosterol than do healthy individuals (35, 36), however, levels of circulatory plant sterols in these individuals are similar to those found in vegetarians (37).

In conclusion, the present study demonstrates that in esterified forms, phytosterol efficacy in total and LDL-cholesterol lowering may be influenced by the saturation state of the plant sterols constituents. Moreover, a mechanism contributing to the action of plant sterols in lowering TC and LDL-C concentrations is through reduction of absorption of dietary cholesterol. Cholesterol biosynthesis is de-suppressed, but not to an extent that plasma cholesterol levels are unaffected. In summary, both esterified β-sitosterol and sitostanol are efficacious in favorably reducing circulating cholesterol concentrations in hyperlipidemic males.

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Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters

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Objective: To examine in humans the effects on serum lipids, lipoproteins and fat-soluble antioxidants of a daily consumption of 2.5 g plant stanols, consumed either once per day at lunch or divided over the three meals.
Design: A randomized, double-blind, placebo-controlled, cross-over design.
Subjects: Thirty-nine healthy normocholesterolemic or mildly hypercholesterolemic subjects participated.
Interventions: Each subject consumed in random order; no plant stanols; 2.5 g plant stanols at lunch; and 2.5 g plant stanols divided over the three meals (0.42 g at breakfast, 0.84 g at lunch and 1.25 g at dinner, which is proportional to dietary cholesterol intake). Each period lasted 4 weeks. Plant stanols were esterified with fatty acids from low erucic rapeseed oil (LEAR) and incorporated into margarines or shortenings.
Results: Consumption of 2.5 g plant stanols at lunch results in a similar low-density lipoprotein (LDL)-cholesterol-lowering efficacy compared to consumption of 2.5 g plant stanols divided over the three meals (−0.29 mmol/l compared with the control period ($P < 0.001$; 95% CI, −0.19 to −0.39 mmol/l) for the once per day diet and −0.31 mmol/l ($P < 0.001$; 95% CI, −0.20 to −0.41 mmol/l)) for the three times per day period). High-density Lipoprotein (HDL) cholesterol and triacylglycerol concentrations did not change. After standardization for LDL cholesterol, the sum of the most lipophylic hydrocarbon carotenoids (ie α-carotene, β-carotene and lycopene) in particular was slightly, though not significantly, lowered by −0.017 ± 0.018 μmol/mmol LDL cholesterol ($P = 0.307$) after the once per day period and by −0.032 ± 0.016 μmol/mmol LDL cholesterol ($P = 0.049$) after the three times per day period.
Conclusions: Our findings suggest that for lowering LDL cholesterol concentrations it is not necessary to consume products rich in plant stanol ester at each meal or simultaneously with dietary cholesterol.
Sponsorship: Raisio Group, Raisio, Finland.
Descriptors: plant stanols; consumption frequency; diet; serum lipids; serum lipoproteins; fat-soluble antioxidants
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Introduction

Plant stanols are useful hypocholesterolemic agents since a daily intake of 2–3 g lowers LDL cholesterol concentrations by 10–15% as found in various populations (Wester, 1999; Law, 2000). The proposed mechanism is that plant stanols reduce the micellar solubility of cholesterol and consequently lower intestinal absorption of both exogenous and endogenous cholesterol (Heinemann *et al*, 1991). This suggests that plant stanol esters should be consumed at each meal to obtain a maximal cholesterol-lowering effect. However, consuming plant stanol esters at lunch and dinner only (Weststrate & Meijer, 1998) showed a decrease in LDL cholesterol comparable to that when consumed three times daily (Miettinen *et al*, 1995; Plat & Mensink, 2000). This suggests that plant stanols are active in the intestinal tract for at least a few hours. It has, however, never systematically been evaluated whether the efficacy of plant stanols to lower serum LDL cholesterol depends on consumption frequency.

The main purpose of the present study therefore was to examine in a normocholesterolemic and mildly hypercholesterolemic population the effects on serum lipids and lipoproteins of a margarine and shortening enriched with plant stanol esters, consumed three times per day, *vs* an equal dose of plant stanol esters, consumed once per day. Also effects on plasma fat soluble antioxidant concentrations were evaluated, as these may be affected by consumption of plant sterol and stanol esters (Weststrate & Meijer, 1998; Gylling & Miettinen, 1999).

Methods

Subjects

Forty-three subjects from Maastricht and surrounding areas applied for the study. Twenty-six of these volunteers had participated in a previous study on the effects of plant stanol esters on serum lipids and lipoproteins (Plat & Mensink, 2000), while the others were recruited via posters in public buildings. Subjects were invited for a screening visit to see if they met our eligibility criteria: age 18–65 y, fasting serum total cholesterol concentration < 6.5 mmol/l

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(251 mg/dl), fasting serum triacylglycerol concentration < 3.0 mmol/l), body mass index < 30 kg/m^2, diastolic blood pressure < 95 mmHg, systolic blood pressure < 160 mmHg, no presence of proteinuria or glucosuria, no use of medication or a diet known to affect serum lipids, and no history of coronary heart disease. Volunteers had not donated blood at least 4 weeks before or during this trial, and did not participate in another biomedical study. All subjects gave their written informed consent before the start of the study. A population of normocholesterolemic and mildly hypercholesterolemic subjects was used, since the serum cholesterol lowering efficacy of plant stanol esters—expressed as a percentage—does not depend on initial serum LDL cholesterol concentrations (Wester, 1999; Law, 2000). Hypercholesterolemic subjects were not included, as many of these patients have a history of cardiovascular disease, or use medication or a diet known to affect serum lipids, which were all exclusion criteria.

One subject was excluded for a serum total cholesterol concentration > 6.5 mmol/l and two subjects decided not to participate. Consequently, the study started with 40 volunteers. One subject dropped out during the first week, because she could not combine the study protocol with her lifestyle. The remaining 39 volunteers, 28 women and 11 men, completed the study successfully. These participants were 31 ± 14 y of age (mean ± s.d.) and had a body mass index of 22.7 ± 2.6 kg/m^2. Before the study started, mean serum total cholesterol and triacylglycerol concentrations were 4.74 ± 0.85 mmol/l (range 2.83–6.28 mmol/l) and 0.99 ± 0.39 mmol/l (range 0.39–1.84 mmol/l) in women and 4.94 ± 89 mmol/l (range 3.37–6.15 mmol/l) and 0.97 ± 0.53 mmol/l (range 0.44–2.02 mmol/l) in men. Seventeen women had cholesterol concentrations below 5.0 mmol/l (normocholesterolemic) and 11 women had cholesterol concentrations between 5.0 and 6.5 mmol/l (mildly hypercholesterolemic). For men, these figures were seven and four, respectively. One man and three women smoked cigarettes, 19 women used oral contraceptives and one woman was postmenopausal.

Design and diets

The study, which was approved by the Medical Ethics Committee of Maastricht University, had a double-blind, placebo-controlled cross-over design (Figure 1). Each subject received three different diets for 4 weeks in one of the six possible treatment orders. There was no washout period between the three different dietary periods. Before the start of the study, the subjects were randomly allocated to one of the six groups. The participants were instructed to maintain their customary lifestyles and home diets throughout the study. During the study, they recorded in diaries any symptoms, visits to physicians, medication used, menstrual phase, alcohol use and any deviation from the protocol. Body weight was recorded weekly.

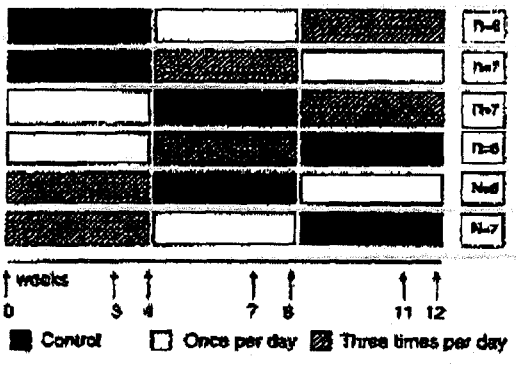

Figure 1 Experimental design of the study.

During the study, the subjects were required to replace at breakfast and at lunch their habitual margarines for an experimental margarine of which, at breakfast 10 g and at lunch 20 g, had to be consumed. Within 1 h after dinner, each participant also had to eat a cake or cookie, which contained 10 g of an experimental shortening. These cakes and cookies were prepared every week by a local bakery especially for this study. To control fat and fatty acid intake as much as possible, each participant also received during each period a shortening without plant stanol esters that had to be used for baking and cooking.

One experimental margarine contained 4.2 g/100 g plant stanols as its fatty acid (low plant stanol ester margarine), and another margarine 12.3 g/100 g (high plant stanol ester margarine). The plant stanol concentration in the experimental shortening was 12.5 g/100 g. Products provided during the control period did not contain any plant stanol esters.

The mixture of vegetable oil and pinewood-derived plant stanols contained approximately 76% sitostanol and 24% campestanol. Sitostanol was prepared from β-sitosterol and stigmasterol, and campestanol from campesterol, both by hydrogenation. Free sitostanol and campestanol were transesterified with rapeseed oil fatty acids, forming fat-soluble sitostanol and campestanol esters. The plant stanol esters were then mixed with the experimental margarines and shortening. The plant stanol esters were added to the experimental margarines at the expense of water and to the experimental shortening at the expense of absorbable fats. All the margarines and shortenings were prepared from low erucic acid rapeseed oil (LEAR) and contained 68% (margarines), 99% (control shortening) or 86% (experimental shortening) absorbable fats. All margarines and the shortening were fortified with normal amounts of vitamin A and D. β-Carotene was used as a coloring agent, while vitamin E was present as a natural compound. The margarines and shortenings were produced and provided by the Raisio Group, Raisio, Finland.

At a daily intake of 10 g margarine at breakfast, 20 g margarine at lunch, and 10 g shortening incorporated into the cakes and cookies after dinner, the aimed plant stanol intake during the experimental periods was 2.5 g. The distribution of plant stanol intake over the day, however, was different (Figure 2). During the once per day period the 2.5 g of plant stanols were consumed once per day at lunch, while during the three times per day period the plant stanols were provided in amounts proportional to cholesterol intake (Ministeries van Welzijn, Volksgezondheid en Cultuur en van Landbouw, Natuurbeheer en Visserij, 1993). Thus, 0.42 g plant stanols were consumed at breakfast, 0.84 g at lunch and 1.25 g at dinner.

The volunteers had to come at least once a week to the Department to receive a new supply of products. The experimental margarines were given in color-labeled tubs, which contained 75 g margarine (breakfast) or 145 g margarine (lunch). The cookies or cakes were provided in similarly color-labeled bags. The tubs and the bags provided margarine, cakes and cookies for one week. Parts of

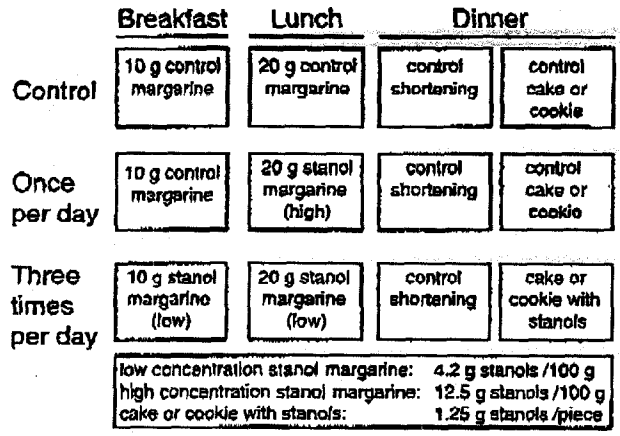

Figure 2 Distribution of plant stanol intake over the day.

all experimental products that were left over at the end of the week had to be returned and were weighed back to calculate the consumption of the experimental margarines and shortening for that week. The shortening without plant stanol esters was packed in a tub of 200 g, which could be used for more than one week.

During the last week of each period, the participants had to fill in a food frequency questionnaire about their eating habits of the previous 4 weeks, in order to estimate their energy and nutrient intakes. Details of the food frequency questionnaire have been published before (Plat & Mensink, 2000). A dietician immediately checked the questionnaires in presence of the subject, for completeness and inconsistencies. Food intake was divided over breakfast, between breakfast and lunch (morning snacks), lunch, afternoon snacks, dinner and evening snacks. Composition of the diets was calculated as described before (Plat & Mensink, 2000).

Blood sampling

Blood was sampled after an overnight fast and after abstinence from drinking alcohol the preceding day and smoking on the morning before blood sampling. All venipunctures were performed by the same person, at the same location and approximately at the same time of the day. No blood was sampled on Mondays. Blood was sampled once at the beginning of the study (day 1) and twice at the end of each dietary period (weeks 3 and 4, 7 and 8, 11 and 12).

A 10 ml clotting tube was always sampled (CORVAC, integrated serum separator tube, Sherwood Medical Company, St Louis, MO, USA). Serum was obtained by low-speed centrifugation at 2000 g for 15 min at 4°C, at least 1 h after venipuncture, and then immediately stored in small portions at −80°C. Serum was used for lipids and lipoprotein analysis. At weeks 0, 4, 8 and 12 blood was also sampled using a 10 ml EDTA tube (Sherwood Medical, Monoject). Plasma was prepared from EDTA blood by centrifuging at 2000 g for 30 min at 4°C. Aliquots were snap-frozen and stored directly at −80°C for analysis of antioxidants. Serum and EDTA blood were also used for analysis of parameters for liver and kidney function, C-reactive protein concentrations and hematological parameters. These parameters were not affected by the diets (Plat & Mensink, 1999).

Chemical analysis

All samples from one subject were analyzed in the same analytical run for total and HDL cholesterol and triacylglycerol concentrations as described before (Plat & Mensink, 2000). The coefficients of variation within runs were 1.9% for serum total cholesterol 2.0% for HDL cholesterol and 3.4% for triacylglycerol. LDL cholesterol concentrations were calculated using the Friedewald equation (Friedewald *et al*, 1972).

Plasma concentrations of tocopherols (α-tocopherol, δ-tocopherol, β + γ-tocopherol), several carotenoids (α-carotene, β-carotene, lycopene, lutein/zeaxanthin, β-cryptoxanthin and phytofluene) and retinol were determined simultaneously, as described (Hess *et al*, 1991; Oostenbrug *et al*, 1997). Briefly, plasma samples were extracted twice with hexane, while retinylacetate was used as internal standard. Antioxidant concentrations were determined by reversed-phase high-pressure liquid chromatography (HPLC). Samples from one subject of weeks 3, 8 and 12 were analyzed in the same analytical run. The mean recovery of retinylacetate was 96.0 ± 7.9%.

Statistical analysis

The data were analyzed with the General Linear Models (GLM) procedure of the SAS program (SAS Institute Inc., 1985). For each subject, lipid and lipoprotein concentrations of weeks 3 and 4, of 7 and 8, and of weeks 11 and 12 were first averaged. The model to examine diet effects included subject, diet, period, carry-over effect and diet × sex as independent variables. Since the carry-over effect, period and the diet × sex interaction term never reached statistical significance, these terms were subsequently omitted from the model. Thus the final model included subject and diet. When the analysis indicated a significant effect of diet ($P < 0.05$), the Tukey method was used to compare the diets pairwise. All values are presented as their means ± standard deviations (s.d.), except in Figure 3, in which values are presented as means ± s.e.

Results

Dietary intakes and body weight

Table 1 shows the estimated daily plant sterol and stanol intakes, as derived from the experimental margarines and shortenings. As expected, total intakes of plant stanols during the once per day diet (2468 ± 173 mg) and during the three times per day diet (2456 ± 121 mg) were significantly higher than those during the control diet ($P < 0.001$). Total plant stanol ($P = 0.672$) and sitostanol ($P = 0.578$) intake was similar during the once per day period and the three times per day period. The slightly higher campestanol intake of 23 mg or 4%, during the once per day period, compared to the three times per day period, was significant ($P < 0.001$). This difference was due to a slight difference in the sitostanol/campestanol ratio of the plant stanol ester mixtures used for the preparation of the low and the high stanol ester margarines.

The daily energy intake and the proportion of energy from the macronutrients and alcohol, as well as cholesterol and fiber consumption, were essentially the same during the three periods of the study. Slight, statistically significant, differences existed in the intakes of fatty acids. This was mainly due to the slightly lower absorbable fat content of the stanol ester shortening compared with the control shortening.

Table 1 Estimated daily intake of plant sterols and plant stanols, energy and nutrients, during the three different diets[a]

	Control period	*Once per day period*	*Three times per day period*
Total plant sterols (mg)[b]	228 ± 44	2729 ± 199*	2682 ± 146*
Of which plant stanols (mg)	0 ± 0	2468 ± 173*	2456 ± 121*
Sitosterol (mg)	112 ± 22	132 ± 22*	123 ± 21**
Sitostanol (mg)	0 ± 0	1867 ± 131*	1879 ± 92*
Campesterol (mg)	81 ± 15	97 ± 15*	82 ± 15*
Campestanol (mg)	0 ± 0	601 ± 42*	578 ± 28*,†
Energy (MJ)	11.0 ± 2.4	11.1 ± 2.5	11.0 ± 2.4
Fat (energy%)	38.8 ± 4.4	38.9 ± 4.1	38.2 ± 4.3
SAFA	13.3 ± 1.9	12.8 ± 1.9	13.5 ± 2.0†
MUFA	16.2 ± 2.2	16.4 ± 2.1	15.7 ± 2.0†
PUFA	7.4 ± 1.3	7.7 ± 1.0	7.1 ± 1.2†
Linoleic acid	5.7 ± 1.1	6.0 ± 0.9	5.5 ± 1.1†
α-Linolenic acid	1.3 ± 0.2	1.3 ± 0.2	1.1 ± 0.2*,†
Cholesterol (mg/MJ)	21 ± 4.5	21 ± 5.9	21 ± 4.5
Protein (energy%)	12.9 ± 1.5	12.7 ± 1.5	12.6 ± 1.4
Carbohydrates (energy%)	45.8 ± 1.2	46.1 ± 4.8	46.7 ± 5.3
Alcohol (energy%)	1.9 ± 1.8	1.6 ± 1.4	1.8 ± 1.9
Fiber (mg/MJ)	2.4 ± 0.5	2.5 ± 0.5	2.5 ± 0.5

[a]Values are means ± s.d. Thirty-nine subjects consumed no plant stanols (control period), 2.5 g plant stanols once a day (at lunch), or 2.5 g plant stanols divided over three meals (0.42 g at breakfast, 0.84 g at lunch and 1.25 g at dinner). Each period lasted 4 weeks. All plant stanols were transesterified with rapeseed oil fatty acids and were administered as its fatty acid esters.
[b]Estimated plant sterol and stanol intake as derived from the experimental margarines and shortenings. Dietary intakes were calculated from food frequency lists filled in during the last week of each period. SAFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
*$P < 0.001$ compared with the control period. **$P < 0.01$ compared with the control period.
†$P < 0.001$ compared with the once per day period. ‡$P < 0.01$ compared with the once per day period.

During the control period, mean estimated daily margarine intake at breakfast was 10.1 ± 0.6 g, and at lunch was 18.8 ± 1.8 g, while the estimated shortening incorporated into the cakes and cookies consumed after dinner was 9.6 ± 0.8 g. For the once per day period, these values were respectively 10.1 ± 0.6, 19.3 ± 1.4 and 9.6 ± 0.8 g, and for the three times per day period respectively 10.2 ± 0.6, 18.8 ± 1.7 and 9.6 ± 0.6 g. Table 2 shows the estimated plant stanol intakes as derived from the margarines and shortening, as well as the cholesterol intakes as divided over breakfast, lunch and dinner, which were all as anticipated. The cakes or cookies prepared with the experimental shortenings were consumed approximately 22 ± 20 min after dinner with no difference between the three periods.

During the different periods of the study, changes in body weight were marginal. At the start of the study mean body weight was 64.5 ± 10 kg for women and 75.2 ± 9 kg for men. At the end of the control period body weight was 64.7 ± 10 kg for women and 75.7 ± 9 kg for men and at the end of the once per day diet and the three times per day diet, mean body weights were 64.2 ± 10 and 64.5 ± 10 kg for women and 75.3 ± 9 and 75.7 ± 9 kg for men, respectively. These values were not significantly different ($P = 0.982$ for the diet of diet for women and $P = 0.993$ for men).

Table 2 Plant stanol and cholesterol intakes at breakfast, lunch and, dinner during the three different diets[a]

	Breakfast	*Lunch*	*Dinner*
Control Period			
Total plant stanols[b] (mg)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cholesterol (mg)	40 ± 31	46 ± 27	122 ± 36
Once per day period			
Total plant stanols (mg)	0.0 ± 0.0	2468 ± 173*	0.0 ± 0.0
Cholesterol (mg)	34 ± 34	50 ± 35	119 ± 41
Three times per day period			
Total plant stanols (mg)	436 ± 26*,†	806 ± 73*,†	1215 ± 69*,†
Cholesterol (mg)	38 ± 32	44 ± 32	113 ± 38

[a]See Table 1.
[b]Estimated plant stanol intake as derived from the experimental margarines and shortenings.
*$P < 0.001$ compared with the control period.
†$P < 0.001$ compared with the once per day period.

Serum lipids and lipoproteins

Table 3 shows that plant stanol ester consumption once a day lowered serum total cholesterol concentrations by 0.32 mmol/l or 12 mg/dl compared with the control period, a reduction of 6.3% ± 6.2% ($P < 0.001$; 95% confidence interval (CI), −0.20 to −0.44 mmol/l). Consumption of a similar amount of plant stanol esters, distributed

Table 3 Fasting lipid and lipoprotein concentrations at the end of the three different diets[a]

	Control period	*Once per day period*	*Three times per day period*
Total cholesterol	5.02 ± 0.88	4.70 ± 0.85*	4.69 ± 0.91*
LDL cholesterol	3.04 ± 0.86	2.74 ± 0.81*	2.73 ± 0.87*
HDL cholesterol	1.50 ± 0.39	1.48 ± 0.41	1.49 ± 0.37
Triacylglycerol	1.05 ± 0.44	1.04 ± 0.45	1.02 ± 0.43
Total to HDL cholesterol ratio	3.6 ± 1.5	3.4 ± 1.4†	3.4 ± 1.4*

[a]See Table 1. Concentrations are expressed in mmol/l, except for the total cholesterol to HDL cholesterol ratio. To convert values for total, HDL and LDL cholesterol to milligrams per deciliter, multiply by 38.67. To convert values for triacylglycerols to milligrams per deciliter, multiply by 88.54.
*$P < 0.001$ compared with the control period. †$P < 0.01$ compared with the control period.

over the day with the three meals, lowered total cholesterol concentrations by 0.33 mmol/l or 13 mg/dl, a reduction of 6.6% ± 7.8% compared with the control period ($P < 0.001$; 95% CI, −0.21 to −0.45 mmol/l). The difference of 0.01 mmol/l for total cholesterol between the once per day period and the three times per day period was not significant ($P = 0.808$; 95% CI, −0.11 to +0.13 mmol/l).

Effects of plant stanol esters on serum total cholesterol were mainly caused by effects on serum LDL cholesterol which were, compared with the control period, significantly decreased by 0.29 mmol/l or 12 mg/dl (−9.4% ± 9.1%; $P < 0.001$; 95% CI, −0.19 to −0.39 mmol/l) after the once per day period and with 0.31 mmol/l or 12 mg/dl (−10.4% ± 11.9%; $P < 0.001$; 95% CI, −0.20 to −0.41 mmol/l) after the three times per day period. As for total cholesterol, the difference of 0.02 mmol/l for LDL cholesterol concentrations between the once per day period and the three times per day period was not significantly different ($P = 0.764$; 95% CI, −0.09 to +0.11 mmol/l). Serum HDL cholesterol and triacylglycerol concentrations were not changed by the diets. Therefore, the total to HDL cholesterol ratios were significantly lower at the end of the once per day period (3.4 ± 1.4; $P = 0.002$) and at the end of the three times per day period (3.4 ± 1.3; $P < 0.001$), compared to the control diet (3.6 ± 1.5).

Fat soluble antioxidants

Consumption of plant stanol esters, either once or three times a day, significantly lowered absolute α-tocopherol and β-carotene concentrations (Table 4). The reduced lycopene and β-cryptoxanthin concentrations nearly reached significance after the once per day period ($P = 0.044$ and 0.032, respectively), while concentrations of both antioxidants were significantly lower after the three times per day period (both $P = 0.001$). In addition, during the three times per day period also phytofluene ($P = 0.008$), and β + γ tocopherol ($P = 0.007$) concentrations were significantly decreased, and changes in lutein/zeaxanthin concentrations nearly reached significance ($P = 0.023$). Retinol concentrations were not affected by plant stanol ester consumption.

Although differences between the once and the three times per day period never reached statistical significance, changes for all antioxidants studied were more pronounced after the three times per day period. Also, changes were larger for the sum of the less polar hydrocarbon carotenoids (ie α-carotene, β-carotene and lycopene) compared with reductions for the sum of the more polar oxygenated carotenoids (ie lutein/zeaxanthin and β-cryptoxanthin) and the sum of the tocopherols, which are more polar than the carotenoids.

After standardization of the antioxidant concentrations for LDL cholesterol (Table 5), none of the antioxidant concentrations was significantly different from the concentrations at the end of the control period. Changes in LDL cholesterol standardized hydrocarbon carotenoids were still slightly negative on the once per day diet (−0.017 ± 0.018 μmol/mmol LDL cholesterol; $P = 0.307$) and −0.032 ± 0.016 μmol/mmol LDL cholesterol ($P = 0.049$) on the three times per day diet. In contrast, after standardization for LDL cholesterol, changes were slightly positive for the oxygenated carotenoids and the tocopherols (Figure 3).

Discussion

Many studies have demonstrated that plant stanol esters, when consumed three times a day with each meal (Miettinen *et al*, 1995; Gylling *et al*, 1997; Plat & Mensink, 2000) or twice a day at lunch and dinner (Weststrate & Meijer,

Table 4 Retinol and fat soluble antioxidant concentrations at the end of the three different diets[a]

	Control period	*Once per day period*	*Three times per day period*
Retinol	2.12 ± 0.41	2.10 ± 0.38	2.14 ± 0.41
δ-Tocopherol	0.21 ± 0.16	0.19 ± 0.09	0.17 ± 0.07
β + γ-Tocopherol	2.67 ± 1.06	2.58 ± 0.95	2.32 ± 0.89[†]
α-Tocopherol	24.40 ± 4.19	23.32 ± 3.78[†]	22.58 ± 3.90[*]
Phytofluene	0.37 ± 0.16	0.34 ± 0.21	0.32 ± 0.20[†]
Lutein/zeaxanthin	0.43 ± 0.15	0.41 ± 0.12	0.40 ± 0.13
β-Cryptoxanthin	0.33 ± 0.12	0.31 ± 0.14	0.30 ± 0.14[†]
Lycopene	0.72 ± 0.28	0.64 ± 0.27	0.60 ± 0.28[†]
α-Carotene	0.05 ± 0.04	0.04 ± 0.03	0.04 ± 0.03
β-Carotene	0.32 ± 0.18	0.26 ± 0.13[*]	0.25 ± 0.13[*]

[a]See Table 1. Concentrations are expressed in μmol/l, except for phytofluene, which is expressed in mV*min/μl (amplification 100).
[*]$P < 0.001$ as compared with the control period. [†]$P < 0.01$ as compared with the control period.

Table 5 LDL cholesterol standardized antioxidant concentrations at the end of the three different diets[a]

	Control period	*Once per day period*	*Three times per day period*
δ-Tocopherol	0.07 ± 0.04	0.07 ± 0.03	0.07 ± 0.03
β + γ-Tocopherol	0.92 ± 0.32	0.96 ± 0.32	0.91 ± 0.36
α-Tocopherol	8.68 ± 2.30	8.91 ± 2.32	9.05 ± 2.51
Phytofluene	0.13 ± 0.06	0.14 ± 0.08	0.12 ± 0.08
Lutein/zeaxanthin	0.15 ± 0.06	0.16 ± 0.07	0.16 ± 0.07
β-Cryptoxanthin	0.12 ± 0.06	0.13 ± 0.08	0.13 ± 0.08
Lycopene	0.25 ± 0.11	0.25 ± 0.12	0.23 ± 0.11
α-Carotene	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02
β-Carotene	0.12 ± 0.07	0.10 ± 0.07	0.10 ± 0.07

[a]See Table 1. Concentrations are expressed in μmol/mmol LDL cholesterol, except for phytofluene which is expressed in mV*min/nmol LDL cholesterol (amplification 100).

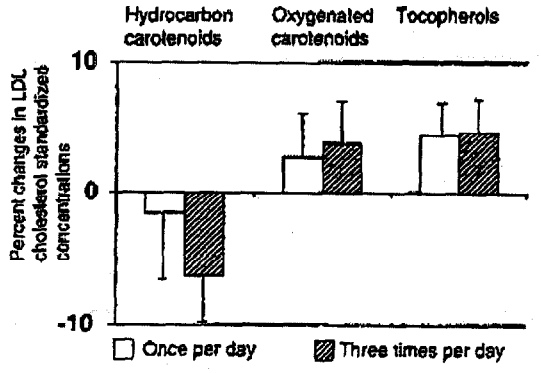

Figure 3 Percentage changes of LDL cholesterol standardized plasma hydrocarbon carotenoid, oxygenated carotenoid and tocopherol concentrations (μmol/mmol LDL cholesterol) at the end of the once per day period and the three times per day period, both compared with the concentrations at the end of the control period (means ± s.e.). Hydrocarbon carotenoids were calculated as the sum of β-carotene, α-carotene and lycopene, oxygenated carotenoids as the sum of lutein/zeaxanthin and β-cryptoxanthin, and tocopherols as the sum of α-tocopherol, β + γ-tocopherol and δ-tocopherol.

1998), lower serum total and LDL cholesterol concentrations. We have now demonstrated that a daily intake of 2.5 g plant stanols as its fatty acid esters, either consumed once per day (at lunch) or divided over three meals (0.4 g at breakfast, 0.8 g at lunch and 1.2 g at dinner), resulted in a similar decrease in serum total and LDL cholesterol. The amount of plant stanols in the latter period was divided over the three meals in such a way that the largest intake was at dinner and the lowest intake at breakfast. This differentiation is largely in correspondence with the distribution of cholesterol intake over the day (Ministeries van Welzijn, Volksgezondheid en Cultuur en van Landbouw, Natuurbeheer en Visserij, 1993; Table 2). Our findings therefore demonstrate that it is not necessary to consume plant stanol ester products simultaneously with dietary cholesterol or with each meal. This provides variety and may increase compliance for potential consumers. Like in other studies, serum HDL cholesterol and triacylglycerol concentrations were not affected. As a result, the total to HDL cholesterol ratio was significantly lower at the end of both the once per day and the three times per day period, as compared with the control period.

The mechanism by which plant stanol esters affect lipoprotein metabolism and lower serum cholesterol concentrations has only partly been elucidated. It is, however, generally assumed that the intestinal absorption of both dietary and biliary cholesterol is reduced in the presence of plant stanols, since the micellar solubility of cholesterol is lowered (Ikeda *et al*, 1989). Therefore, it has been suggested that plant sterols, which also lower the micellar solubility of cholesterol, should be consumed at each cholesterol-containing meal to achieve an optimal effect (Mattson *et al*, 1982). However, this suggestion is not supported by our findings. We therefore hypothesize that plant stanols, or plant stanol esters, remain in the intestinal lumen or in the enterocytes for a while. Indeed, only 70% of an orally administered single bolus of ^{14}C labeled sitostanol to male Wistar rats is found in the feces after 24 h (Ikeda & Sugano, 1978). After 2 and 3 days the cumulative fecal excretions were 90% and 97%, respectively. Thus, when the low absorption of sitostanol into the circulation (Hassan & Rampone, 1979) is neglected, at least 25–30% of the sitostanol is still in the intestinal tract after one day. However, when rats were fed 0.5% cholesterol and 0.5% sitostanol (W/W) for 18 days, the daily fecal excretion of sitostanol showed a recovery of approximately 100% (Sugano *et al*, 1977). This implies that in rats, at least within 18 days, a steady state was reached and sitostanol intake equaled sitostanol excretion. This still does not elucidate whether sitostanol remains in the intestinal lumen, and if so, in which part, or in the enterocytes. It also does not answer the question of how long plant stanols are active in the intestine. Studies with caco-2 cells have addressed the question whether micellar ^{14}C-labeled sitosterol could be taken up in the enterocyte and subsequently be excreted across the basolateral membrane (Field *et al*, 1997). To our knowledge no such studies with sitostanol have been published. It appeared that sitosterol was indeed associated with the caco-2 cells. It was, however, not esterified intracellular and not excreted to the basolateral medium. This implies that sitosterol can indeed remain in or can be associated with enterocytes. The functional significance of these findings, however, is unknown. Theoretically sitosterol could remain associated with the enterocytes only temporarily, be released into the lumen after several hours, and consequently affect micellar solubility of intestinal cholesterol at that moment. It can, however, also be speculated that plant sterols or stanols not only affect micellar solubility of cholesterol, but have additional effects on intestinal lipoprotein metabolism as well.

In this study, serum LDL cholesterol concentrations were significantly reduced by 9–10%, when plant stanol esters were consumed. In a previous study, also in a normocholesterolemic and mildly hypercholesterolemic population, serum LDL cholesterol concentrations decreased by 11–13%, when 3.8 or 4.0 g plant stanols as its fatty acid esters were consumed (Plat & Mensink, 2000). As already discussed (Mensink & Plat, 1998; Wester, 1999), hardly any additional benefit is obtained when daily intake of plant stanols exceeds 2.2 g.

Although total fat consumption during the three diet periods was similar, the fatty acid compositions of the diets were not entirely comparable. This was due to the slightly lower absorbable fat content of the stanol ester shortening compared with the control shortening. However, the marginal differences in the dietary fatty acid compositions were too small to have a major impact on serum lipoproteins. The LDL-cholesterol-lowering effect of the once per day period might have been overestimated by 0.02 mmol/l compared with the control period, while the LDL-cholesterol-lowering effect of the three times per day diet might have been underestimated by 0.01 and 0.04 mmol/l, when compared with the control period and the once per day period, respectively (Mensink & Katan, 1992).

Consumption of 2.5 g plant stanols three times a day significantly lowered most of the carotenoid and tocopherol isomers studied. In contrast, consumption of a similar amount of plant stanols once day at lunch only resulted in reduced absolute α-tocopherol and β-carotene concentrations. In addition, all antioxidants studied showed slightly lower concentrations at the end of the three times per day period compared with the concentrations at the end of the once per day period (Tables 4 and 5). These absolute reductions can be explained largely by a reduced number of LDL particles in the circulation, which are major carriers of the fat-soluble antioxidants. Therefore, the differences were no longer significant after standardization for LDL choles-

terol. Furthermore, we have shown that, in particular, the most lipophylic hydrocarbon carotenoid concentrations (ie α-carotene, β-carotene and lycopene) were lowered by plant stanol ester consumption. The mechanism and the biological significance of these effects, however, remain to be elucidated.

From our results we conclude that a daily consumption of 2.5 g plant stanols as fatty acid esters either at lunch or divided over the three meals does not affect its serum LDL-cholesterol-lowering efficacy. This implies that it is not necessary to consume plant stanol esters simultaneously with dietary cholesterol or with each meal. We therefore hypothesize that plant stanols, or plant stanol esters, remain in the intestinal lumen, or possibly in or associated with the enterocytes. It can also be speculated that plant stanols not only affect micellar solubility of cholesterol, but have other intestinal effects on lipoprotein metabolism as well. Therefore, further research will be necessary to elucidate the mechanism by which plant stanols lower LDL cholesterol.

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